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Cholic Acid Attenuates ER Stress-Induced Cell Death in Coxsackievirus-B3 Infection

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Coxsackievirus Type B3 (CVB3) is an enterovirus that belongs to the *Picornaviridae* and causes various diseases such as myocarditis and hand-foot-mouth disease. However, an effective antiviral drug is still not developed. In this study, we looked for potential inhibitors of CVB3 replication by examining the survival of CVB3-infected HeLa cells. We detected an antiviral effect by cholic acid and identified it as a candidate inhibitor of CVB3 replication. Cholic acid circulates in the liver and intestines, and it helps the digestion and absorption of lipids in the small intestine. HeLa cells were cultured in 12-well plates and treated with cholic acid (1 and 10 μg/ml) and 10^6 PFU/ml of CVB3. After 16 h post-infection, the cells were lysed and subjected to western blot analysis and RT-PCR. The production of the viral capsid protein VP1 was dramatically decreased, and translation initiation factor eIF4G1 cleavage was significantly inhibited by treatment with 10 μg/ml cholic acid. Moreover, cholic acid inhibited ERK signaling in CVB3–infected HeLa cells. RT-PCR showed that the amounts of the CVB3 RNA genome and mRNA for the ER stress-related transcription factor ATF4 were significantly reduced. These results showed that cholic acid strongly reduced ER stress and CVB3 proliferation. This compound can be developed as a safe natural therapeutic agent for enterovirus infections.

Keywords: Coxsackievirus B3, ER stress, cholic acid, proliferation, myocarditis

Introduction

Coxsackievirus Type B3 (CVB3) is a member of the enterovirus group of the *Picornaviridae* and causes acute and chronic myocarditis, which is also known as inflammatory cardiomyopathy because of heart muscle inflammation [1, 2]. The typical symptoms are difficulty in breathing, chest pain, decreased ability to exercise, and irregular heartbeat. Acute myocarditis may not initially lead to mortality, but it may cause dilated cardiomyopathy [3–5]. In addition, the replication and persistence of CVB3 directly affects cardiac damage and disease [6]. Therefore, targeting CVB3 prevents not only damage to the heart but also immune responses by preventing viral spread. CVB3 replication is directly processed by viral protein synthesis, and virus-encoded protease cleavage of the viral polyprotein into structural and nonstructural proteins [7]. Based on the life cycle of these viruses, therefore, we conducted antiviral tests against CVB3 by screening natural compounds from marine microbes.

Cholic acid is synthesized from cholesterol in the liver with chenodeoxycholic acid [8, 9]. These two major bile acids are roughly equal in concentration in humans [10–12]. In our previous study, we reported the antiviral effect of *Fructus Amomi Cardamomi* extract against CVB3 [13]. To develop new drugs against enteroviruses, we tested the ability of purified natural compounds to modulate signaling and inhibit viral replication.

The endoplasmic reticulum (ER) is involved in protein synthesis, folding, quality control, distribution, and

degradation. When a misfolded protein accumulates in the ER, ER stress occurs. Viral infections also lead to ER stress, followed by the induction of apoptosis or autophagy [14, 15].

In this study, we screened new candidate anti-enterovirus drugs from marine microbial natural products. Cholic acid inhibited proliferation of CVB3 in HeLa cells and inhibited cell signaling involved in enterovirus proliferation. In addition, cholic acid inhibited viral protease 2A and ERK signaling, and viral RNA amplification. These results suggest that cholic acid has potential as an antiviral agent with no cell toxicity and is a strong candidate to be developed as an anti-enterovirus drug.

Materials and Methods

Virus and Cell Lines

HeLa cells were monolayer cultured on a 100-mm dish. After 16 h, the medium was replaced and virus stock was added. When the CPE reached 90% or more after the virus infection, the dish was completely frozen at −80°C. Freezing and thawing was repeated three times [11]. Then, the supernatant obtained from centrifugation at 448 ×*g* for 10 min was filtered and stored at −80°C. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum at 37°C in a CO_2 incubator [16].

Purification of Single Compound

The strain SNA-042 was isolated from marine sediment from Choupori sinan goon, Jeollanam-do, South Korea. It was identified as a member of the genus *Streptomyces* by 16S rRNA sequence analysis. SNA-042 was cultivated in 40 L of 2.5 L Ultra Yield Flasks, each containing SYP medium (10 g/l of soluble starch, 4 g/l of yeast, 2 g/l of peptone, 10 g/l of $CaCO_3$, 20 g/l of KBr, 8 g/l of $Fe_2(SO_4)_3 \cdot 4H_2O$) dissolved in 250 ml of distilled water and 750 ml of natural seawater at 25°C with shaking at 150 rpm. After 7 days, the aqueous layer was extracted with ethyl acetate. The solvent was removed under reduced pressure to yield 1.5 g extracts of SNA-042. The crude extract was fractionated by flash column chromatography on silica gel, and eluted with a gradient mixture of CH_2Cl_2/CH_3OH to obtain eight fractions. Fraction 7 was purified by reversed-phase HPLC (Phenomenex Luna C18(2), 250 × 100 mm, 2.0 ml/min, 5 µm, UV = 200 nm) using an isocratic solvent system (H_2O:CH_3CN = 50:50) to obtain cholic acid (8.0 mg).

Compound Screening

We performed antiviral compound screening to select an effective compound. First, HeLa cells cultured on a 96-well plate were infected with 20 µl of virus (CVB3 10^6 pfu/ml) per well. The drug was diluted to 1/10 from 10 µg/ml to 100 pg/ml, respectively. After 16 h, the HeLa cells status of the (+) portion was confirmed with 8 µl of Cell Counting Kit 8 (CCK-8), a cell growth detection reagent [17].

Cholic Acid Treatment

To confirm the cell signaling, we treated purified cholic acid to HeLa cells, cultured on a 12-well plate. First, 30 µl of virus was inoculated into each well except for the negative (control) sample. Then, we added 500 µl of cholic acid diluted from 100 µg/ml to 1 µg/ml in 5% FBS-containing DMEM. After 16 h infection, 8 µl of CCK-8 was added and incubated for 2 h [17].

Western Blot Analysis

HeLa cells were lysed using 1× PBS lysis buffer (1× PBS, 5 mM EDTA, 0.5% Triton X-100). Protein was extracted and mixed with sample buffer to perform western blot analysis. Aliquots of total cell extracts were loaded onto a 10% acrylamide gel. After electrophoresis, the bands were transferred to a Hybond-ECL PVDF membrane. The membranes were blocked with 5% non-fat dry milk blocking buffer and incubated with eIF4G1 (1:1,000, rabbit polyclonal antibody), p-ERK (1:1,000, mouse monoclonal antibody), total-ERK, and enteroVP-1 antibodies (1:1,000, mouse monoclonal antibody) [18, 19]. All data were quantified by NIH-imageJ software [11].

Reverse Transcription PCR

To confirm the amplification of the CVB3 RNA genome, total RNA was isolated with TRIzol reagent (Invitrogen, USA). We performed reverse transcription PCR by using a Maxime Reverse-Transcription (RT) kit (Intron Biotech, Inc., Korea) with 2 µl of the aliquot of the total extract as template. To amplify the positive strand of the virus, we performed the RT reaction to synthesized cDNA. After that, we performed PCR with VP1-antisense primer (5′-GTCAGCATGCGTGTACTTTA-3′) and VP1-sense primer (5′-GCGAAGAGTCTATTGAGCTA-3′) as the cDNA template. The PCR product was electrophoresed on 1.5% agarose gel [17]. All data were quantified by NIH-ImageJ software [11].

Statistical Analysis

All data were expressed as the mean ± SD. The results of Control and Virus Infection groups were analyzed by Student's *t*-test by Prism 3.0. Statistically, $p < 0.05$ was considered as significant.

Results

Effective Compound Selection

The antiviral effect against CVB3 was verified by analyzing the survival of HeLa cells. We tested various compounds identical to those naturally present in the ocean plant. HeLa cells were infected with CVB3 and the antiviral effect was examined by using six concentrations of the tested compounds ranging from 100 pg/ml to 10 µg/ml. The survival rate of HeLa cells was increased by more than 90% by 10 µg/ml cholic acid compared with untreated sample (positive) (Fig. 1A). Moreover, cholic acid toxicity was confirmed by HeLa cell survival assay to be in a dose-

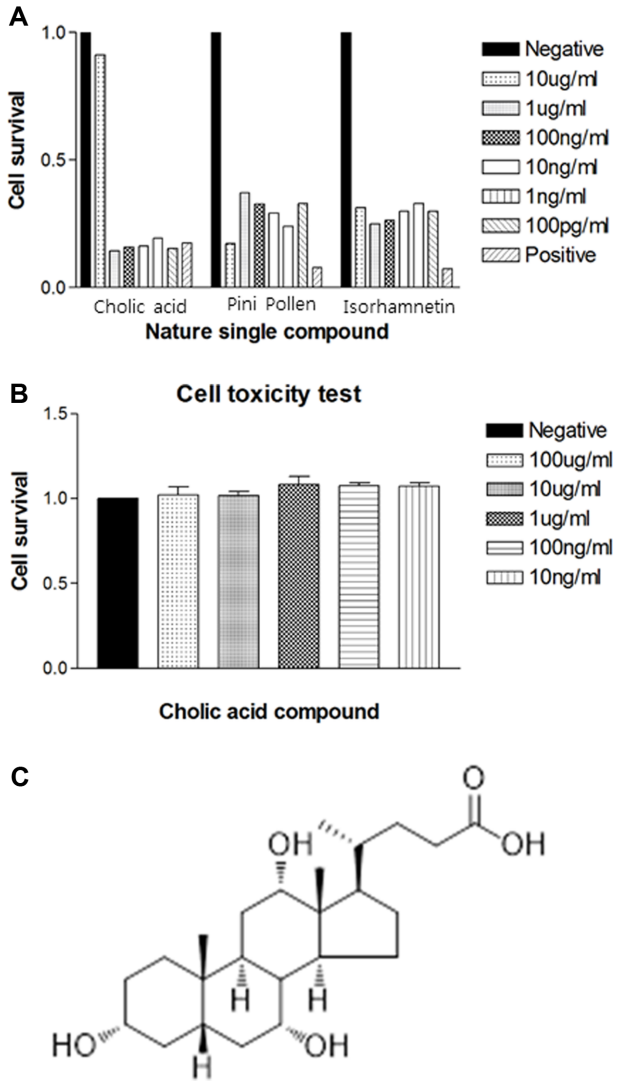

Fig. 1. Screening of anti-enteroviral natural compounds in HeLa cells.

(**A**) The antiviral effect of each compound was tested by cell survival assay in CVB3 infection. Negative: without virus infection; Positive: virus infection only. (**B**) Cholic acid cytotoxicity was confirmed by CCK-8 cell survival assay. (**C**) Chemical structure of cholic acid.

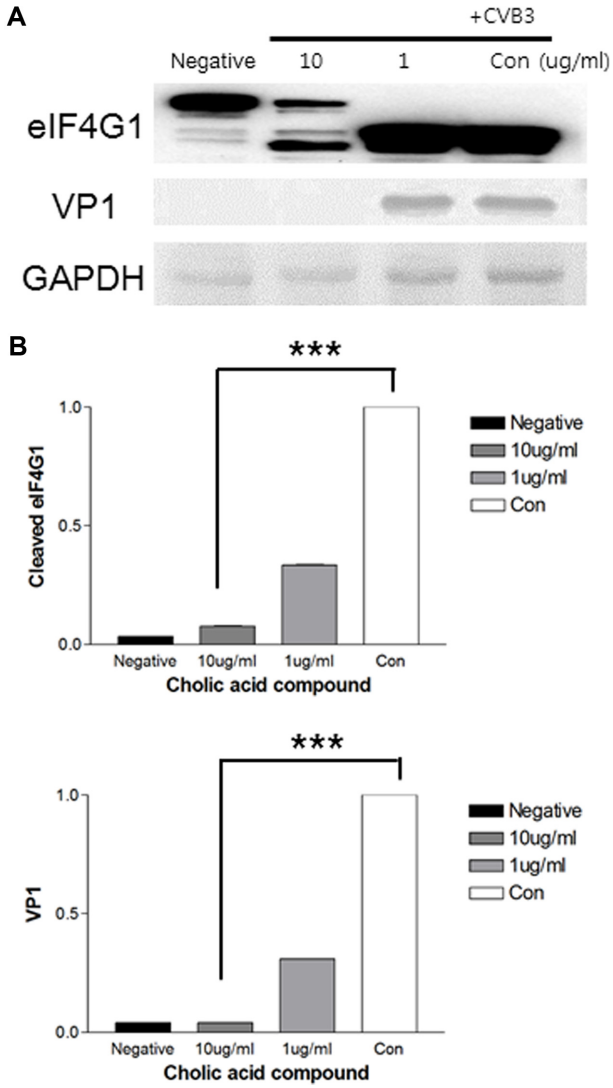

Fig. 2. Cholic acid inhibits CVB3 replication through protease 2A inhibition.

(**A**) Cholic acid was added to HeLa cells following CVB3 infection. Virus protease 2A-induced eIF4G1 cleavage and capsid protein VP1 expression were dramatically reduced by cholic acid in a dose-dependent manner (10 μg/ml). (**B**) Western blot results were quantitated by NIH-ImageJ. ***, $p < 0.001$.

dependent manner. There was no observed cell death from cholic acid treatment (Fig. 1B).

Inhibition of CVB3 Protein Production

The expression of VP1, the capsid protein of CVB3, was reduced by 100% after treatment with 10 μg/ml cholic acid. This concentration of cholic acid inhibited CVB3 replication and CVB3 protease 2A production. It inhibited the transcription initiation factor eIF4G1 cleavage induced by CVB3 protease 2A (Fig. 2). Using CVB3 with inserted green fluorescent protein (CVB3-GFP), we visualized CVB3 proliferation. HeLa cells were cultured as described above, infected with CVB3-GFP with cholic acid, and observed under a fluorescence microscope (Fig. 3A). CVB3-GFP-infected HeLa cell proteins were extracted and GFP expression was identified by western blot analysis. GFP expression was significantly suppressed in a dose-dependent

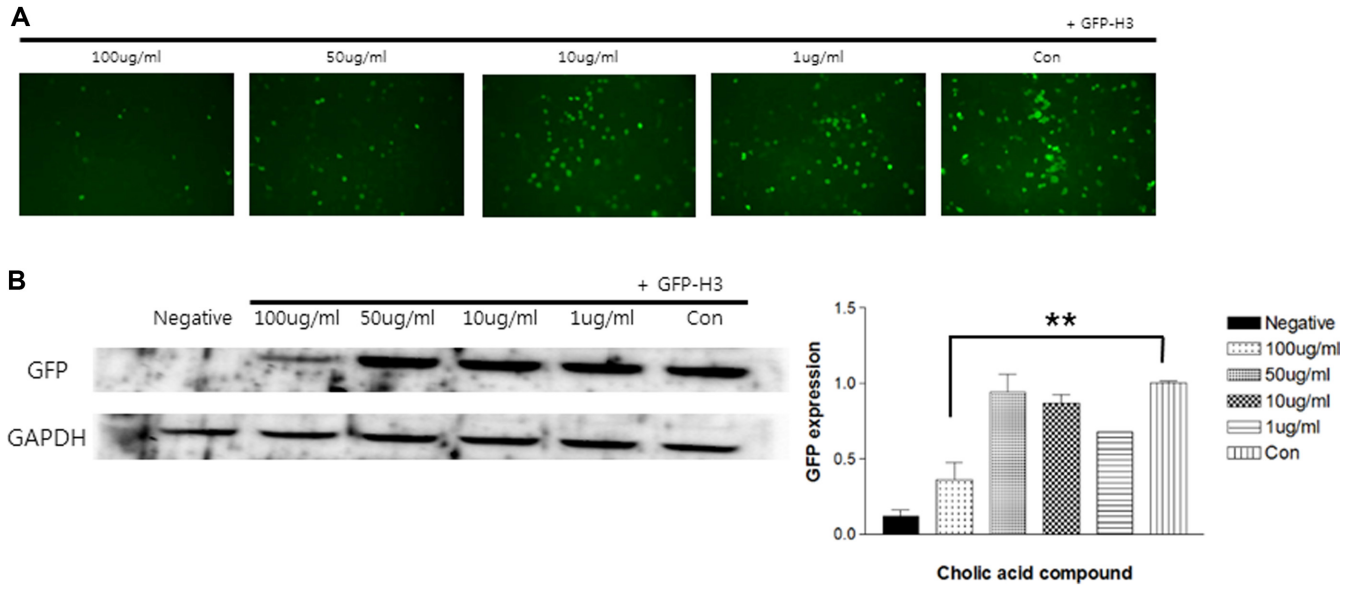

Fig. 3. Cholic acid inhibits CVB3 protein production.
(**A**) Cholic acid was added to HeLa cells following CVB3-GFP infection. The degree of fluorescence was decreased depending on the cholic acid concentration. (**B**) Western blot results were quantitated by NIH-ImageJ. **, $p < 0.01$.

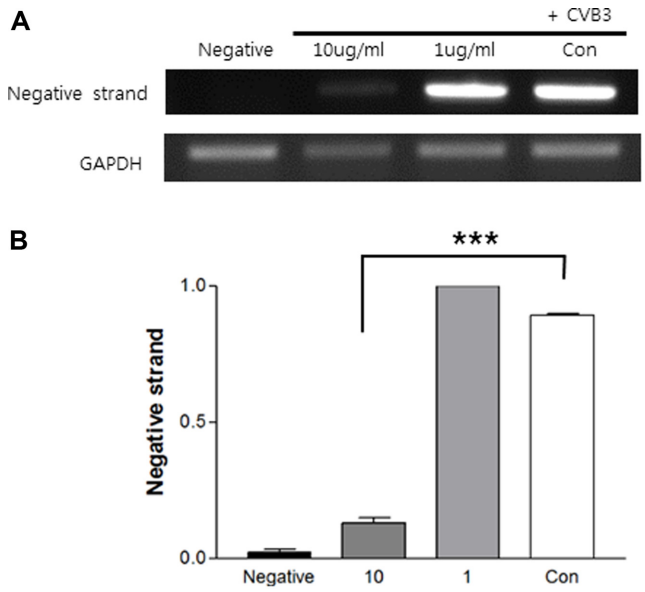

Fig. 4. Cholic acid inhibits CVB3 RNA genome amplification.
(**A**) CVB3 RNA numbers were quantified by RT-RCR. Positive-strand RNA was significantly reduced by cholic acid treatment (10 μg/ml). (**B**) All data were quantified by NIH-ImageJ. ***, $p < 0.001$.

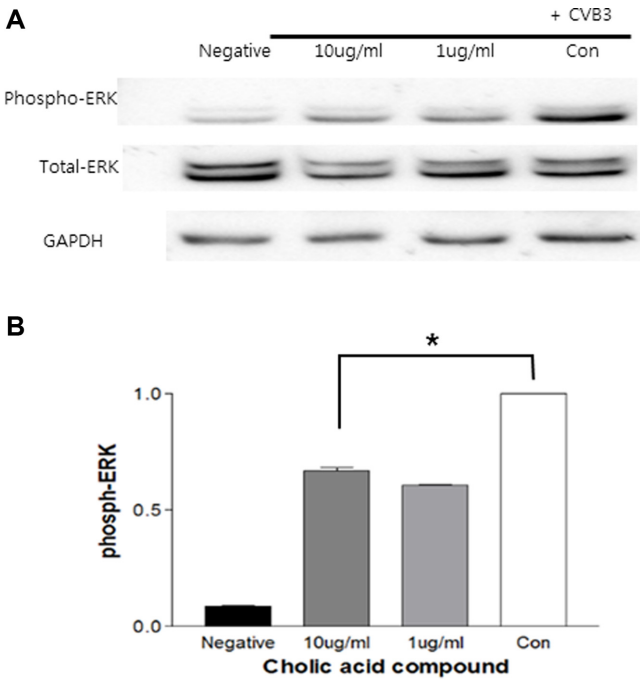

Fig. 5. Cholic acid inhibits early replication of CVB3.
(**A**) ERK phosphorylation was dramatically decreased by cholic acid treatment. (**B**) Data are presented as the mean of three independent experiments. All data were quantitated by NIH-ImageJ. *, $p < 0.05$.

manner after cholic acid treatment (Fig. 3B).

Cholic Acid Inhibits CVB3 Gene Amplification

To investigate whether cholic acid affected the amplification of the CVB3 gene, HeLa cell monolayers were treated with cholic acid as described above, and total RNA was extracted

and used for reverse transcription with primers for the CVB3 VP1 gene to examine the amplification of the negative-strand RNA of the virus. CVB3 is a positive-strand RNA virus, and production of the negative strand is essential for viral gene replication. Treatment with cholic acid strongly inhibited CVB3 gene replication (Fig. 4). These results show that generation of new viruses was effectively suppressed by blocking viral gene replication in cholic acid-treated CVB3-infected cells.

Cholic Acid Suppresses MAPK Signaling in CVB3 Infection

In previous studies, enteroviruses have been reported to increase the activity of ERK and AKT, which mediate signaling required for CVB3 proliferation after cell infection. The cholic acid cell signal regulation in CVB3 infected HeLa cells was confirmed by western blotting to determine the activity of ERK (pERK) and to determine whether there was a correlation with CVB3 replication. Despite proliferation of the virus, the level of pERK decreased when cells were treated with 10 μg/ml cholic acid compared with the untreated sample (Con) (Fig. 5). These results confirmed that treatment with cholic acid inhibited proliferation of the virus by inhibiting ERK activation and that cholic acid could help maintain cell viability.

Cholic Acid Suppresses ER Stress in CVB3 Infection

CVB3 infection induced ER stress and cell death for virus proliferation. We evaluated the RNA expression of ATF4, an ER stress regulatory gene, with cholic acid treatment in CVB3 infection HeLa cells. ATF4 RNA expression was significantly decreased by 100 μg/ml cholic acid treatment (Fig. 6). These results strongly support that cholic acid inhibited CVB3 replication and preserved cell survival in CVB3 infection.

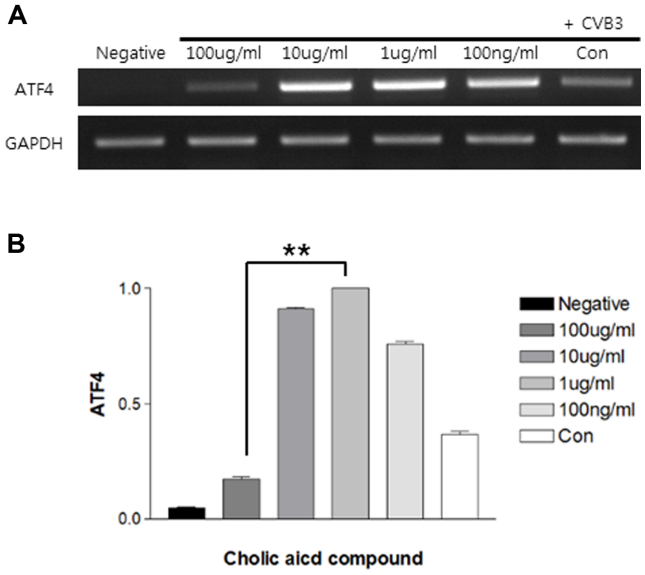

Fig. 6. Cholic acid suppreses ER stress-regulating gene ATF4 in CVB3 infection.

(**A**) Cholic acid treatment dramatically suppressed gene ATF4 expression. (**B**) All data were quantitated by NIH-ImageJ. **, $p < 0.01$.

Discussion

Enteroviruses have a gene structure and life history similar to those of characterized polioviruses, and the same method of vaccine development has been used for both groups. However, effective vaccines for enteroviruses have not been developed [20]. In this study, we tried to develop a new antiviral agent from among purified compounds identical to those found in natural ocean plants. These compounds are structurally identical to those present in nature, but the problem is that when absorbed into the body the functions may not be the same. It is also not well known whether natural substances play a role in the body in any mechanisms. Nonetheless, when absorbed into the body, purified natural compounds are expected to be significantly less cytotoxic than other synthetic compounds.

Viral myocarditis is an inflammatory process that causes damage to cardiac muscle cells [21]. We tested the inhibitory effect of cholic acid in CVB3 infection. We found that treatment with cholic acid significantly suppressed CVB3 infection and cell mortality. We also demonstrated that cholic acid reduced damage to HeLa cells by inhibiting virus capsid protein (VP1) production. The cleavage of eIF4G1, a transcription initiation factor that is cleaved by viral protease 2A, was significantly decreased by cholic acid treatment. In addition, CVB3-GFP was used to infect HeLa cells to confirm the proliferation of live viruses [11]. CVB3 is a positive single-strand RNA virus, and production of a negative-strand RNA is essential for viral genome amplification. Cholic acid treatment strongly inhibited negative-strand RNA amplification of CVB3. Enterovirus replication occurs through the activation of ERK, a cell signaling molecule [22, 23]. ERK activity was significantly reduced following the drug treatment and the cells remained viable. One of the important factor for cell survival in CVB3 infection is ER stress regulation, which is associated with the onset of neural tube defects caused by diabetes [14, 15, 24]. Cholic acid reduced ER stress in CVB3-infected cells and is therefore expected to be effective in the treatment of enterovirus infection. However, the mechanism is still unclear. We need more study to define the mechanisms

of correlation between ER stress reduction and CVB3 replication inhibition by cholic acid treatment.

In conclusion, we found that cholic acid inhibits ERK cell signaling and ER stress associated with replication and proliferative effects of CVB3, a major causative agent of viral myocarditis. We expect cholic acid may possibly be used to develop a new therapeutic agent against enterovirus.

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