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Coxsackievirus A16 infection triggers apoptosis in RD cells by inducing ER stress

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ABSTRACT

Coxsackievirus A16 (CA16) infection, which is responsible for hand, foot and mouth disease (HFMD), has become a common health problem in Asia due to the prevalence of the virus. Thus, it is important to understand the pathogenesis of CA16 infection. Viruses that induce endoplasmic reticulum (ER) stress are confronted with the unfolded protein response (UPR), which may lead to apoptotic cell death and influence viral replication. In this study, we found that CA16 infection could induce apoptosis and ER stress in RD cells. Interestingly, apoptosis via the activation of caspase-3, -8 and -9 in the extrinsic or intrinsic apoptotic pathways in RD cells was inhibited by 4-phenyl butyric acid (4PBA), a chemical chaperone that reduces ER stress. These results suggest that CA16 infection leads to ER stress, which in turn results in prolonged ER stress-induced apoptosis. This study provides a new basis for understanding CA16 infection and host responses.

1. Introduction

Coxsackievirus A16 (CA16), one of the major causative agents of human hand, foot and mouth disease (HFMD), is a positive single-stranded RNA virus [1]. Recent reports examining CA16 have mainly focused on the epidemiology of HFMD [2,3], the detection or characterization of CA16, and the comparisons between CA16 and enterovirus 71 (EV71) [4]. However, despite the established association of CA16 infection with HFMD, the pathogenic mechanisms of its viral infection are still unclear. Therefore, it is critical to study the pathogenesis of CA16.

The endoplasmic reticulum (ER) is a critical organelle in all eukaryotic cells and is responsible for viral replication and maturation. There is a high protein load in virus infected cells, and extreme or prolonged ER stress can lead to the activation of the unfolded protein response (UPR) and even apoptosis [5]. The relationship between virus infection and ER stress has recently been identified, and it has been suggested that some viruses are able to regulate ER stress [6,7]. For example, studies of the Flaviviridae family showed that these viruses use the ER to complete viral replication, and this process can result in ER stress, leading to an increased susceptibility to apoptosis [8]. The ER chaperone 78 kDa glucose-regulated protein (GRP78) and the three major signaling components, inositol-requiring ER-to-nucleus signal kinase 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK), cope with the temporal and developmental variations in the ER. As a downstream molecule of IRE1, c-Jun amino-terminal kinases (JNK) is also associated with apoptosis. While a lot is known about ER stress, our understanding of CA16-induced apoptosis and the relationship between CA16 and ER stress is incomplete.

Although some recent studies have shown that picornaviruses could induce the apoptotic pathway involving caspase-9 and caspase-3 [9], these studies failed to observe the extrinsic apoptotic pathway, which is associated with the activation of caspase-8. Some studies have shown that some viruses can induce, and even regulate ER stress [6,7], but they neglected to investigate the consequences to the host cells and how the virus-induced ER stress results in those consequences.

We used RD cells as a model to conduct our research as these cells represent an interesting model for CVA16 biology [10]. The results of our study showed that CA16 infection triggers ER stress by inducing all three signaling pathways and that CA16 infection efficiently induced apoptosis in RD cells. Treatment with 4-phenyl butyric acid (4PBA), a chemical chaperone that reduces ER stress, abrogated the loss of mitochondrial membrane potential (MMP)

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and the activation of the caspases in CA16 infection. These data indicated that CA16 triggers both the intrinsic and extrinsic apoptotic pathways in RD cells and that ER stress might be responsible for the induction of apoptosis in CA16-infected RD cells.

2. Materials and methods

2.1. Virus and cell lines

CA16 was obtained from the China Center for Type Culture Collection (CCTCC); this sample was isolated from the throat swab specimens of HFMD patients in the Hubei province. RD cells were purchased from the American Type Culture Collection (ATCC). Coxsackievirus A16 was cocultured with the RD cells in MEM supplemented with 100 U/mL penicillin (Sigma–Aldrich), 100 μg/mL streptomycin (Sigma–Aldrich) and 10% fetal bovine serum (FBS; Gibco) in petri dishes (Costar Corp.) at 37 °C in a humidified atmosphere containing 5% CO_2. Coxsackievirus A16 virus stocks were harvested from the infected RD cell supernatants 7 days after infection.

2.2. Flow cytometry analysis

Flow cytometry was performed on an Epics Altra II flow cytometer (Beckman Coulter), and $>10^6$ cells were analyzed per sample. Data analysis was conducted using the EXPO32 V1.2 analysis software. Annexin V assays were used to detect cells with externalized phosphatidylserine on the outer leaflet of the cell membrane, which is indicative of early apoptosis. RD cells were infected with CA16 for 24 h at an MOI of 5. The cells were then harvested by gentle trypsinization and stained with 1 μg/mL FITC-conjugated Annexin V (eBioscience) and 10 μg/ml propidium iodide (eBioscience). The samples were then analyzed by flow cytometry.

2.3. Cell viability assay

RD cells were cultured in 96-well plates in medium with or without CA16 infection at an MOI of 5 and/or other treatments. The plates were incubated at 37 °C with 5% CO_2 for 24 h and then subjected to a CCK8 assay using the CCK8 assay kit (Beyotime), according to the manufacturer's instructions.

2.4. Western blot analyses

The cells were harvested and homogenized in RIPA buffer containing a protease inhibitor cocktail (Promotor). After two freeze–thaw cycles, the samples were centrifuged at 12,000 rpm for 15 min at 4 °C. Protein quantification was carried out using a BCA protein assay (Thermo). Equal amounts of soluble proteins were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane by electroblotting; electrotransfer was performed at 4 °C for 100 min at 273 mA in Tris/glycine buffer with 20% methanol. Western blot analysis was performed using 1:1000 dilutions of the following primary antibodies: GRP78 (EPITOMICS), ATF6 (Abcam), CHOP (Cell Signaling Technology), IRE1α (Cell Signaling Technology), p-PERK (Cell Signaling Technology), caspase-3 (EPITOMICS), caspase-8 (EPITOMICS), caspase-9 (EPITOMICS), JNK (EPITOMICS), BAX (Cell Signaling Technology), p-JNK (EPITOMICS) or GAPDH (Abcam). All incubations occurred overnight at 4 °C. After probing with 1:10,000 diluted HRP-conjugated secondary antibodies (Proteintech Group) for 1.5 h at room temperature, the protein bands were detected using the ECL SuperSignal West Pico Trial Kit (Pierce). Protein loading was normalized using an anti-GAPDH antibody (Abcam).

2.5. XBP1 splicing analysis

Total RNA was isolated from the infected cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich), according to the manufacturer's instructions. The RNA samples were treated with RNase-free DNase I (Promega) to eliminate any genomic DNA contamination. The integrity and quantity of the extracted RNA was analyzed by measuring the absorbance at 260/280 nm on a Lambda 25 spectrophotometer (Perkin Elmer). The DNase-treated total RNA was reverse-transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions. The PCR reaction was then performed with following primers specific for XBP1: forward, 5′-CTGGAACAGCAAGTGGTAGA-3′; reverse, 5′-CTGGGTCCTTCTGGGTAGAC-3′. The PCR product was then treated with the restriction enzyme PstI (Promega), and the resulting fragments were run on an agarose gel.

2.6. Measurement of caspase-3 activity

The caspase-3-like protease activity in the lysate was measured using a colorimetric caspase-3 assay kit (Beyotime) according to the manufacturer's protocol. Briefly, the 100 μl reaction mixture contained 30 μl of the cell lysate and 10 μl of the caspase-3 substrate (200 μM) in assay buffer, and the assay was carried out in a 96-well plate. To account for non-specific hydrolysis of the substrate, a control reaction mixture containing 30 μl of cell lysate and 10 μl of 20 μM caspase-3 inhibitor in assay buffer was generated. Both mixtures were incubated for 90 min at 37 °C, and the absorbance was read at 405 nm. The caspase-3 activity of each sample was calculated following the manufacturer's instructions.

2.7. Statistical analysis

The results are presented as the mean ± SD. Comparisons between two groups were determined using a Student's *t*-test, whereas comparisons between multiple groups were carried out using a one-way ANOVA. Differences were considered statistically significant when the *p*-value was less than 0.05.

3. Results

3.1. CA16 induces apoptosis in RD cells

To identify the host cell response during CA16 infection, we were interested in testing whether CA16 infection induced apoptosis. CA16 infection did result in RD cell death after 48 h of incubation, as confirmed by a time-dependent decrease in cell viability (Fig. 1A) and the observed morphological changes, such as cell shrinkage, rounding and detachment from the surface of the plate (Fig. 1B). To determine the manner of cell death that was occurring in the CA16-infected RD cells, we investigated the activity of caspase-3, a key enzyme involved in apoptosis, in RD cells and used Annexin V staining to confirm apoptosis. As most of the infected RD cells were dead between 48 and 72 h after infection, we chose an earlier time point (24 h) to evaluate the apoptotic markers. CA16 infection was associated with an increase in the number of cells displaying active caspase-3 (Fig. 1C). Accordingly, Annexin V staining, which detects the presence of the apoptotic marker phosphatidylserine, showed that the proportions of early (Annexin V + PI−) and late (Annexin V + PI+) apoptotic cells were significantly increased in the CA16-infected cells compared to the control cells ($p < 0.01$, Fig. 1D). These data demonstrated that CA16 induces apoptotic death in cultured RD cells.

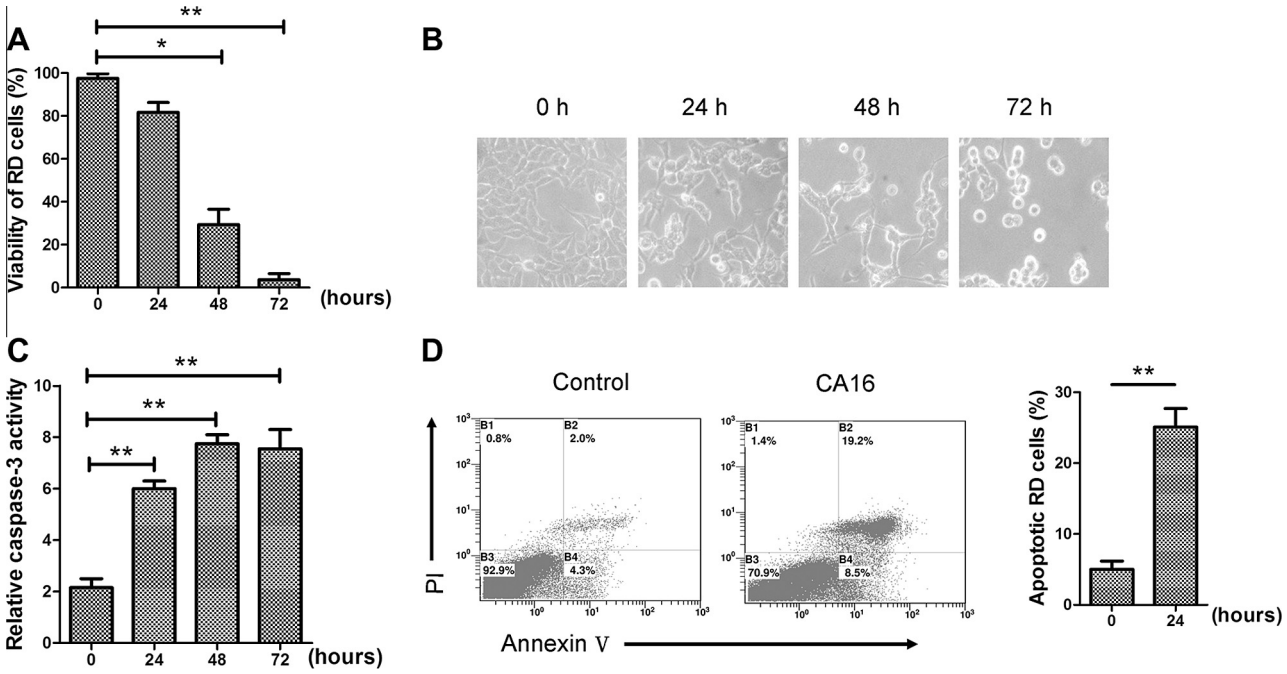

Fig. 1. CA16 induces apoptosis in RD cells. (A) RD cells were infected with CA16 (MOI = 5) at 0 h, and CCK8 analysis was performed to investigate the viability of the RD cells 24, 48 and 72 h after infection. The results show that the viability of the infected RD cells was reduced following CA16 infection in a time-dependent manner. As of 48 h post-infection, the viability of the infected RD cells was significantly decreased compared to the cells at 0 h. $^{*}p < 0.05$. The cell viability was obviously decreased at 72 h compared to 0 h. $^{**}p < 0.01$. (B) The morphology of the infected RD cells was investigated by microscopy at the same time points in (A). As of 48 h post-infection, the RD cells had shrunk, rounded and detached from the surface of the dish. As of 72 h post-infection, there were almost no RD cells that maintained a normal shape. (C) The activity of caspase-3 in the infected RD cells was also detected at the same time points. Compared to 0 h post-infected or uninfected RD cells, there was a significant increase in the activity of caspase-3 in the infected RD cells after 24 h or more. $^{**}p < 0.01$. (D) There was also a significant increase in the proportion of both early (Annexin V + PI−) and late (Annexin V + PI+) apoptotic RD cells following infection with CA16 for 24 h compared to 0 h post-infected or uninfected RD cells. $^{**}p < 0.01$.

3.2. Caspase activation is associated with CA16-induced apoptosis in RD cells

To further confirm that apoptosis was induced by CA16, apoptosis inhibition experiments were performed. z-VAD-fmk is a pan-caspase inhibitor that blocks apoptosis by inhibiting almost all of the caspases in the cell. Following treatment with z-VAD-fmk, the CA16-induced cell death of RD cells was inhibited, as observed by microscopy (Fig. 2A). The cell viability of the CA16-infected RD cells was significantly increased following treatment with z-VAD-fmk ($p < 0.05$, Fig. 2B). Annexin V staining was also conducted to determine the effects of z-VAD-fmk on the CA16-infected RD cells. As shown in Fig. 2C, z-VAD-fmk significantly, but not completely, decreased the proportion of apoptotic cells in the CA16-infected RD cells ($p \leqslant 0.05$).

3.3. CA16 triggers ER stress in RD cells

Because GRP78 works as a master regulator of ER stress and interacts with three ER stress mediators, IRE1, ATF6 and PERK, the over-expression of GRP78 is a marker of ER stress as its expression is induced by ER stress. To identify whether CA16 infection was associated with ER stress, Western blot analysis was performed to detect GRP78 expression. When RD cells were infected with CA16 for 24 h, there was an increase in GRP78 expression (Fig. 3A), strongly suggesting that CA16 triggers ER stress in RD cells. We also detected the activation of the three ER stress mediators. The results show that the phosphorylations of IRE1, ATG6 and PERK were increased in RD cells that were infected with CA16 for 24 h (Fig. 3B and C). In response to ER stress, these three mediators function to reduce the levels of the new proteins translocated into the ER lumen, thus enhancing the protein-folding capacity and secretion potential of the ER, and to facilitate the transport and degradation of ER-localized proteins. The pathways downstream of these three mediators were also observed. JNK is a target of IRE1 and can be phosphorylated by activated IRE1 to mediate apoptosis. We found that the phosphorylation of JNK was increased in the CA16-infected RD cells using immunoblot analysis (Fig. 3B). We also found that C/EBP homologous protein (CHOP), a common molecule downstream of ATG6 and PERK that promotes the transcription of apoptosis-associated genes, was up-regulated in the CA16-infected RD cells (Fig. 3C).

To further confirm the association between CA16 infection and ER stress, a second marker of ER stress, spliced XBP1 mRNA, was analyzed. Total RNA was isolated from the RD cells and reverse transcribed into cDNA. Primers specific for XBP1 were then used to amplify the XBP1 splice variants. The splice variants and their PCR products differ by only a few base pairs, and this difference is difficult to resolve on an agarose gel. Therefore, the amplified DNA products were digested with the restriction enzyme Pst I, which cleaves a site that is only present in the unspliced XBP1 mRNA. Spliced XBP1 RNA cannot be cleaved by Pst I and appears as a higher molecular weight band. CA16 infection increased the splicing of XBP1 in the RD cells (Fig. 3D).

3.4. The CA16-induced apoptotic cell death of RD cells is associated with ER stress

Because the above results indicated that CA16 infection induced both ER stress and apoptosis, we attempted to explore the connection between ER stress and apoptosis in RD cells. The extrinsic apoptotic pathway refers to apoptosis initiated by the death receptors that activate caspase-8. Many stimuli, including viruses, are able to activate this apoptotic pathway. We investigated the effects of CA16-induced ER stress on the activation of caspase-3 and caspase-8 by treating the cells with 4PBA. The results showed that

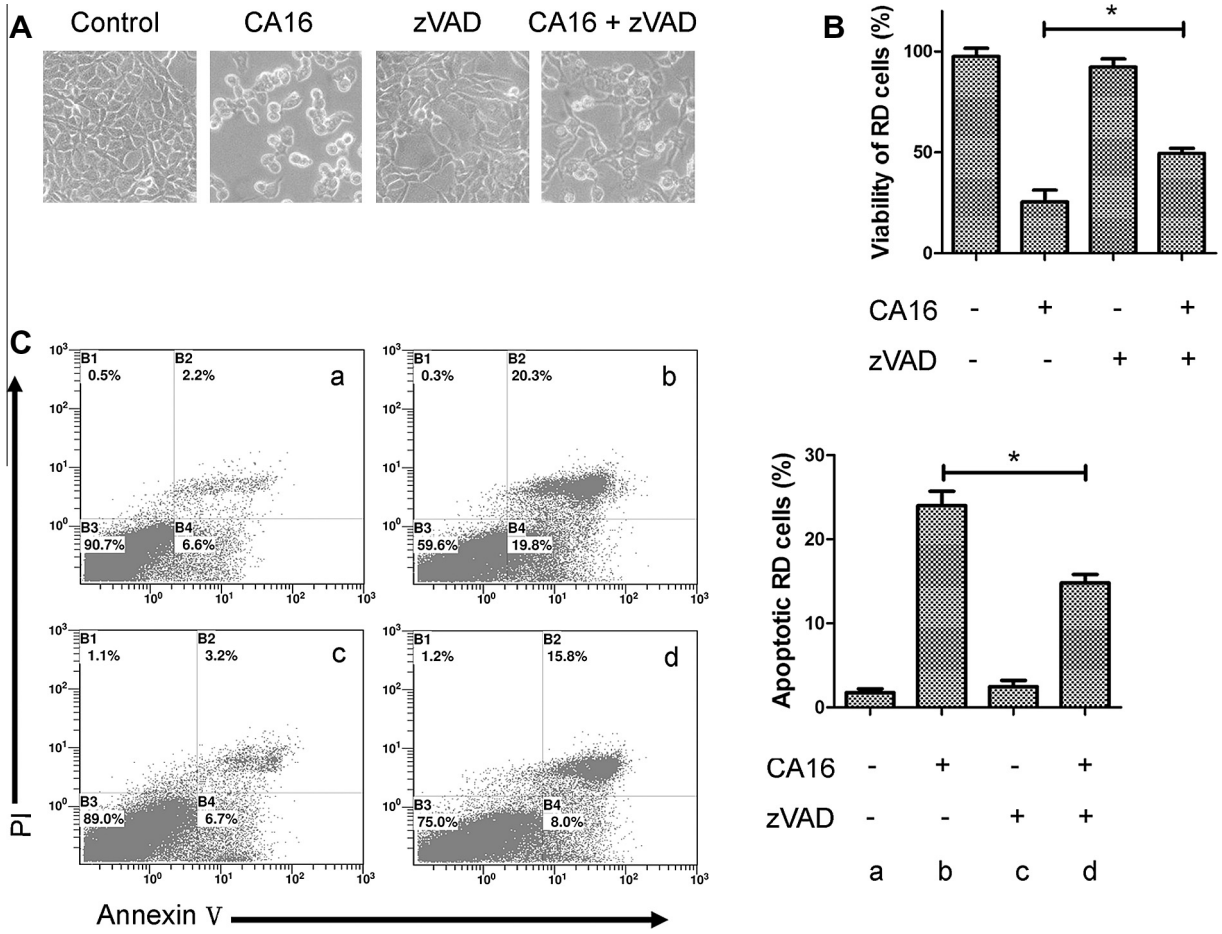

Fig. 2. The pan-caspase inhibitor z-VAD-fmk partially inhibits the apoptosis of the CA16-infected RD cells. (A) Following treatment with 200 μM z-VAD-fmk and infection with CA16 (MOI = 5) for 24 h, the morphological changes in the cells were observed by microscopy, and the results showed that the mortality of the RD cells decreased compared to the CA16-infected RD cells without z-VAD-fmk. (B) The results of the CKK8 analysis showed that there was a significant increase in cell viability in the 200 μM z-VAD-fmk treated CA16-infected RD cells compared to the untreated, infected cells. $^{*}p < 0.05$. (C) Annexin V staining showed that treatment with 200 μM z-VAD-fmk resulted in a significant decrease in apoptosis in the CA16-infected RD cells. $^{*}p < 0.05$.

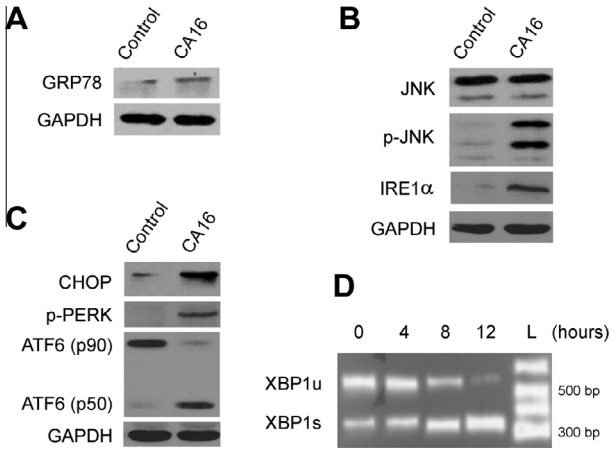

Fig. 3. CA16 induces ER stress in RD cells. Western blot analysis showed that (A) CA16 infection (MOI = 5) increased the amount of GRP78 in RD cells 24 h post-infection. (B) The level of IRE1α and the phosphorylation of its downstream molecule, JNK, were up-regulated in the CA16-infected RD cells. (C) Two other ER stress sensors, ATF6 and PERK, were both phosphorylated following CA16 infection, and the level of their common downstream molecule CHOP increased accordingly, leading to apoptosis-associated gene transcription. (D) XBP1 analysis showed that the amount of spliced XBP1 was also increased in the CA16-infected RD cells 24 h post-infection.

the inhibition of ER stress reduced the activation of caspase-3 and caspase-8 (Fig. 4A). Additionally, caspase-3 activity in the CA16-infected RD cells was decreased following treatment with 4PBA ($p < 0.05$, Fig. 4B). The intrinsic apoptotic pathway involves the loss of MMP, resulting in the release of cytochrome c and the activation of caspase-9. To investigate the MMP of the infected RD cells, the TMRM dye was used. A loss of MMP occurred in the CA16-infected RD cells, and this loss could be rescued by treatment with 4PBA ($p < 0.05$, Fig. 4C). Western blot analysis was used to detect the expression of caspase-9, and we found that the levels of active caspase-9 were increased post-infection but were reduced following treatment with 4PBA. The main checkpoint in the mitochondrial apoptotic pathway is the ratio of the pro-apoptotic (BAX) and anti-apoptotic (Bcl2) members of the Bcl2 family. Here, our results showed that CA16 infection up-regulated the expression of BAX and down-regulated the expression of Bcl-2, and treatment with 4PBA had the opposite effect on BAX and Bcl-2 expression in the infected RD cells (Fig. 4D). These results suggested that CA16-induced apoptosis results from ER stress through both the extrinsic and intrinsic apoptosis pathways.

4. Discussion

In this study, we found that both ER stress and apoptotic cell death occurred in RD cells following CA16 infection. Additionally,

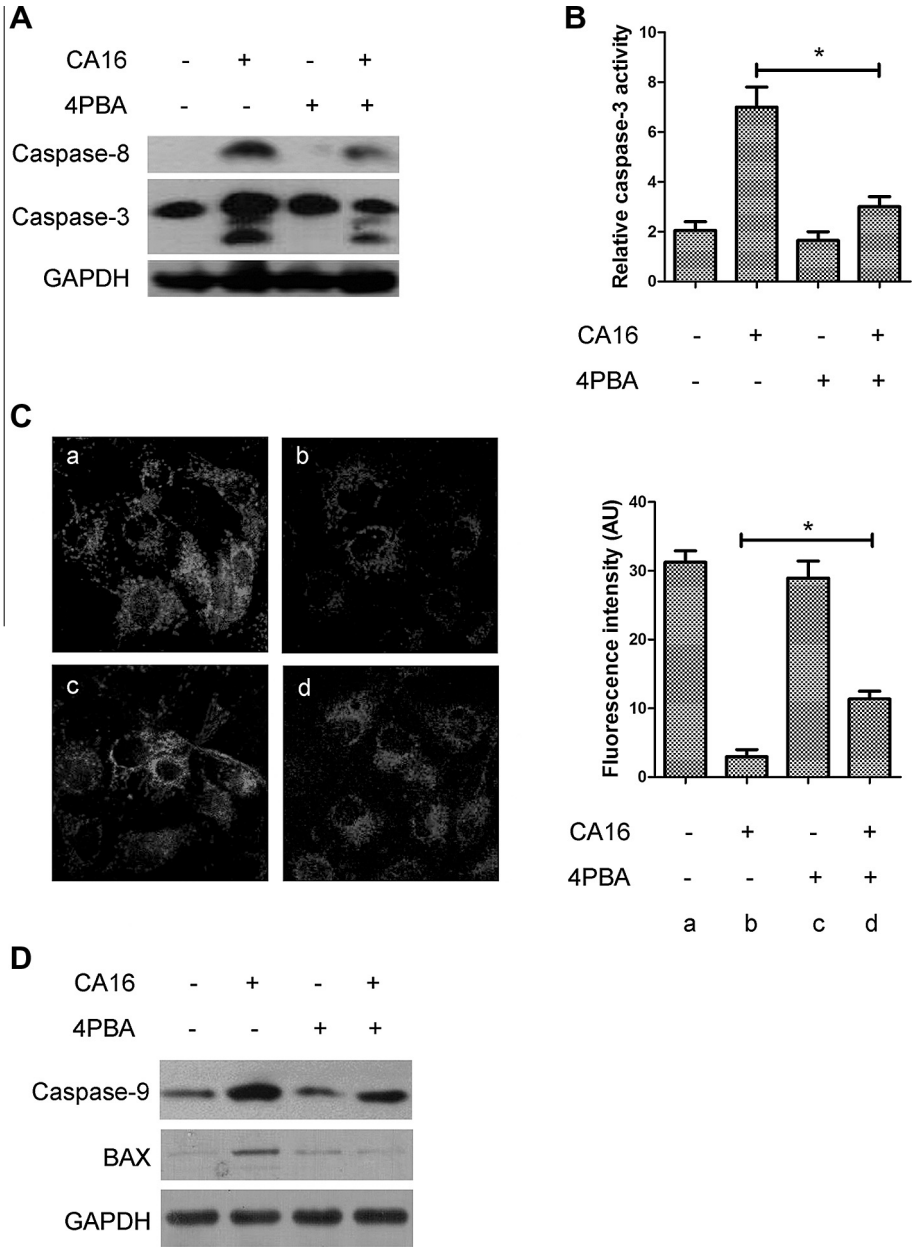

Fig. 4. The CA16-induced apoptotic cell death of RD cells is associated with ER stress. (A) The ER stress inhibitor 4PBA inhibited the cleavage of caspase-8 and -3 in the RD cells that were infected with CA16 for 24 h at an MOI of 5. (B) At 24 h post-infection (MOI = 5), the caspase-3 activity of the CA16-infected RD cells was significantly decreased following treatment of 10 mM 4PBA. $^{*}p < 0.05$. (C) TMRM was used to measure the mitochondrial membrane potential. The fluorescence intensity of the CA16-infected RD cells was significantly decreased following treatment with 10 mM 4PBA compared to the untreated CA16-infected cells. $^{*}p < 0.05$. (D) Treatment with 10 mM 4PBA also resulted in a decrease in BAX expression and reduced cleavage of caspase-9 in the CA16-infected RD cells.

the ER stress triggered by CA16 infection was responsible for the initiation of apoptosis in the RD cells.

Infection with single stranded RNA viruses, such as EV71 and pneumoviruses, induces apoptosis through different signaling pathways [11,12]. Although CA16 is closely related to EV71, little attention has been paid to CA16, in contrast to the increased interest in EV71; this is most likely because CA16 causes only mild symptoms [13]. In our study, we show that CA16 triggers apoptosis in RD cells. CA16 infection activated caspase-3, -8, and -9 in RD cells, and the CA16-induced apoptosis could be inhibited by the pan-caspase inhibitor z-VAD-fmk in this study. The activation of caspase-8 by CA16 indicated that CA16 infection could activate the extrinsic apoptosis pathway in RD cells.

Mitochondrial dysfunction is believed to participate in apoptosis and has even been suggested to be crucial to the apoptotic pathway [14]. The loss of MMP is a main feature of mitochondrial dysfunction. The depolarization of the MMP is induced by the opening of the mitochondrial permeability transition pore (MPTP), which results in the release of apoptotic factors and the loss of oxidative phosphorylation [14,15]. The released cytochrome c binds to Apaf-1, which then associates with procaspase-9 to activate this caspase cascade [16]. Using the TMRM dye, an acute decrease in MMP was observed in the CA16-infected RD cells in this study. In addition to facilitating the opening of MPTP, CA16 infection was also able to activate caspase-9 and caspase-3. The increase in the amount of Bax in the RD cells strongly suggested that CA16 infection facilitated the opening of the MPTP through Bax. This suggests that CA16 infection can also trigger the intrinsic apoptotic pathway in RD cells.

Viral infection of mammalian cells induces endoplasmic reticulum stress during the synthesis of the viral polypeptides and the

replication of the viral genome [17]. If the infection eventually endangers the cells, the cell may undergo autophagy or apoptosis to restrict the replication and spread of the virus [17,18]. GRP78 is an ER-resident chaperone and a master regulator of ER stress [19,20]. The expression of GRP78 and CHOP is most commonly used to evaluate ER stress in cells. Accumulating evidence suggests that one or more viral proteins trigger BiP expression during viral infection, particularly in cells infected with paramyxoviruses, such as simian virus 5 and respiratory syncytial virus [21,22]. Additionally, infection of cells by other RNA viruses, such as flaviviruses, also increases GRP78 expression [8]. Here, we found that GRP78 and CHOP increased in expression following CA16 infection, which suggests the occurrence of ER stress in RD cells.

During the early phase of ER stress, protective mediators outweigh the pro-apoptotic mediators, such as CHOP. If these steps fail to reestablish homeostasis, the IRE1 and ATF6 signals are attenuated, resulting in an imbalance in which the pro-apoptotic mediators overwhelm the pro-survival mediators [23]. When CHOP is expressed in mammalian cells, it facilitates apoptosis [24]. An interaction occurs between IRE1 and tumor necrosis factor receptor-associated factor 2 (TRAF2) results in the recruitment and activation of downstream JNK, which is involved in a variety of pro-apoptotic signaling pathways [25]. The ATF6 cytosolic fragment is a transcription factor responsible for the transcriptional induction of XBP1 and many ER chaperone-encoding genes [26]. In our study, the level of IRE1 increased in the CA16-infected RD cells, and CA16 infection promoted the splicing of XBP1. We also found that the phosphorylation of JNK increased following CA16 infection. Besides, CA16 infection promoted the conversion of p90-ATF6 to p50-ATF6, which suggests that the activation of ATF6 also occurs following CA16 infection. The third transducer, PERK, was activated by undergoing phosphorylation in the CA16-infected RD cells.

More interestingly, our study showed that CA16 infection-induced ER stress was probably associated with the apoptosis that was triggered by CA16 in RD cells. Following the treatment with 4PBA, the activities of caspase-8, caspase-9 and caspase-3 were partly inhibited, and the loss of MMP was abrogated. At the same time, the expression of Bax decreased.

In summary, CA16 infection activated caspase-3, -8, and -9, resulting in the activation of both the extrinsic and intrinsic apoptosis pathways in RD cells, and this apoptosis was partially inhibited by the pan-caspase inhibitor z-VAD-fmk. Meanwhile, CA16 infection induced a loss of MMP and an increase in BAX expression in RD cells. Additionally, our results demonstrated that the expression levels of GRP78 and CHOP were increased following infection with CA16, while the three ER stress transducers were all activated, as was the splicing of XBP1 and the phosphorylation of JNK. The activation of both the extrinsic and intrinsic pathways is involved in the CA16 infection-induced ER stress and is a partial mechanism of RD cell apoptosis.

Programmed cell death could be an effective way for viruses to spread and induce pathogenesis. Therefore, interesting questions remain, and further investigations of the molecular relationships between the virus, ER stress and apoptosis may yield important information.

Acknowledgments

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