Enterovirus 71 induces apoptosis by directly modulating the conformational activation of pro-apoptotic protein Bax

Xiaodong Han¹ and Haolong Cong²,*

Abstract
Enterovirus 71 (EVA71), a virus of the genus Enterovirus in the family Picornaviridae, is one of the main causative agents of hand, foot and mouth disease in infected infants and young children. In this study, we report that cells with EVA71 infection exhibit increased levels of cytochrome c release and caspase-3 activation. EVA71 infection induces the conformational activation of pro-apoptotic protein Bax and the subsequent formation of oligomers of Bax in mitochondria. Inhibitors that block caspase-8 activation cannot inhibit apoptosis induced by EVA71 infection. Importantly, cells with Bax but not Bak or caspase-8 knockdown show resistance to apoptosis induced by EVA71 infection. Mitochondria isolated from EVA71-infected cells display clear Bax-binding ability and the subsequent release of cytochrome c. Therefore, these results indicate that EVA71 infection directly impacts the mitochondrial apoptotic pathway by modulating the recruitment and activation of Bax.

INTRODUCTION
Apoptosis is a highly regulated form of programmed cell death that contributes to the elimination of damaged, aged or virally infected cells. A variety of key events in apoptosis are related to mitochondria, including the release of caspase activator cytochrome c (Cyt c), loss of mitochondrial trans-membrane (TM) potential and altered cellular oxidation-reduction [1, 2]. The mitochondrial apoptotic pathway usually involves a variety of pro- and anti-apoptotic proteins of the Bcl-2 family which are united by the presence of at least one of four conserved Bcl-2 homology (BH) domains [3]. The anti-apoptotic proteins Bcl-2, Bcl-w, Mcl-1, Bfl-1 and Bcl-XL contain all four BH domains (BH1–4) [4, 5]. The pro-apoptotic proteins PUMA, Bim, Bid, Bad, Bik, Noxa and Bmf containing only a single BH3 domain (BH3 only) are often responsible for conveying the initial death signal [6]. The pro-apoptotic Bcl proteins Bak and Bax possess BH1 to BH3 and are required for the induction of apoptosis via the mitochondrial pathway [7].

In most cells, Bax is normally localized in the cytosol or loosely associated with the outer mitochondrial membrane (OMM), whereas Bak is mostly localized in the OMM and remains inactive in non-apoptotic cells [8]. Upon activation, both Bak and Bax undergo extensive conformational changes that result in the exposure of an N-terminal epitope and subsequent homo-oligomerization [9, 10]. Bax and Bak homo-oligomers are thought to form pores that permeabilize the mitochondrial outer membrane and allow the release of pro-apoptogenic factors [10]. In normal cells, Bak and Bax are functionally blocked by the anti-apoptotic Bcl-2 family members [5]. Studies indicated that Bak has a hydrophobic groove serving as a receptor for a BH3 protein; the groove is occluded by the C-terminal TM domain, thus keeping Bak in the cytoplasm in the form of inactive monomers [11]. Following some cytotoxic stimulation, Bax is activated and undergoes a series of conformational changes, leading to Bax translocation to the mitochondria, oligomerization and integration into the mitochondrial membranes and subsequent apoptosis [12]. Bak resides on the OMM in association with Mcl-1 and Bcl-XL, which occupy the dimerization and killing domain BH3 of Bak [13]. Upon activation, Bak is released from Mcl-1 and Bcl-XL, and the BH3 domains are displaced for oligomerization, which promotes cell death [5].

Because of Bcl-2 proteins and mitochondrial permeability transition pore complex playing a central role in regulating the fate of an infected cell, many viruses have evolved strategies that modulate their activity [14]. For example, the regulation of mitochondrial membrane potential by viral protein R of human immunodeficiency virus type 1 is facilitated by Bax binding to adenine nucleotide translocator,
whereas it is prevented by Bcl-2 overexpression and permeability transition pore complex inhibitors [15]. In addition, human immunodeficiency virus type 1 protease processes procaspase-8, which cleaves Bid to its activated form (tBid) [16]. Severe acute respiratory syndrome coronavirus protein 7A contributes to virus-induced apoptosis by inhibiting Bcl-X<sub>L</sub> [17].

The apoptotic mitochondrial pathway in Poliovirus (PV)-infected neuronal IMR5 cells is Bax dependent. It is reported that the mitochondrial pathway of apoptosis mediated by activation and cleavage of caspase-9 is the main pathway in enterovirus 71 (EVA71)-induced apoptosis [18]. However, the detailed mechanism of EVA71-induced apoptosis still remains to be elucidated. In this study, we demonstrate that EVA71 infection impacts the mitochondrial apoptotic pathway by directly altering Bax conformation and triggering its oligomerization and redistribution from the cytosol to mitochondria.

**RESULTS**

**EVA71 activates mitochondrial apoptotic pathway in HeLa cells**

To determine if EVA71 infection could impact the mitochondrial apoptotic cascades, we initiated our studies by monitoring the ability of EVA71 infection to release Cyt c and activate caspase-3. HeLa cells were infected with EVA71 strain BrCr, strain HuBei09, strain AnHu09 and EVA71 (UV) (strain BrCr treated by ultraviolet). By annexin V-FITC staining, apoptotic cells were detected at the indicated time post infection. A significant proportion of cells infected with EVA71 strain BrCr, strain HuBei09 and strain AnHu09 but not EVA71 (UV) - or mock-infected cells displayed increased annexin V fluorescence intensity (Fig. 1a). In addition, the annexin V fluorescence signal arising was accompanied with an increased time of EVA71 infection (Fig. 1a), indicating that EVA71 infection induced cell apoptosis. To determine if caspases are crucial to EVA71-induced apoptosis in HeLa cells, we examined the effects of caspase inhibitors: zDEVD.fmk, a wide-spectrum caspase inhibitor that irreversibly inhibits caspase-3, as well as other proteases including caspase-6, caspase-8 and caspase-10; zLEHD.fmk, an irreversible inhibitor of caspase-9. Cells treated with the zDEVD.fmk or zLEHD.fmk displayed a very low level of annexin V fluorescence signal, indicating that the apoptosis was completely inhibited, and cytolysis induced by EVA71 infection was directly dependent on caspase activation (Fig. 1b). In addition, when compared with EVA71-infected cells treated with DMSO, no obvious change of annexin V fluorescence signal was observed in EVA71 mock- or EVA71 (UV)-infected cells, indicating that apoptosis induced by EVA71 depends on the activation of caspases.

To further assess the ability of EVA71 to initiate mitochondrial apoptotic pathway in HeLa cells, we monitored the Cyt c release and the caspase-3 or caspase-9 activation in cells infected with EVA71. The cytoplasmic and mitochondrial fractions were separated, and Western blot was performed to determine the level of Cyt c in EVA71 (BrCr)- and EVA71 (UV)-infected cells. Results showed that cells treated with staurosporine (an initiator of apoptosis by activating caspase-3) or infected with EVA71 (BrCr) displayed a dramatical loss of Cyt c from the mitochondria fraction to the cytosolic fraction, while no Cyt c was detected in the cytosolic fraction of EVA71 (UV)- or mock-infected cells (Fig. 1c). The activation of caspase-3 and caspase-9 in cells was also analysed; Western blot analysis showed that the activation of both caspase-3 and caspase-9 was significantly increased with the extension of infection time. Forty-eight hours post infection, lower molecular weight bands that represent the active forms of caspase-3 and caspase-9 could be detected in EVA71-infected cells (Fig. 1c). It implied that EVA71 (BrCr) infection directly resulted in the proteolytic processing of caspase-3 and caspase-9 inactive zymogen into activated fragments; especially, almost no full-length caspase-3 can be detected in EVA71 (BrCr)-infected cells (Fig. 1c). However, no obvious Cyt c loss and caspase-3 or caspase-9 activation were observed in cells infected with EVA71 (UV) (Fig. 1c). The loss of Cyt c from mitochondria was often accompanied with the loss of inner mitochondrial membrane potential. Thus, to analyse the effect of EVA71 (BrCr) on mitochondrial membrane potential, cells were infected with EVA71 and stained with tetracycline rhodamine ethyl ester (TMRE). Mitochondrial membrane potential was then measured by flow cytometry at the indicated times post infection. DMSO was used as a vehicle control, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a positive control to measure the background fluorescence of mitochondria that were unable to maintain a potential gradient. Cells infected with EVA71 showed a clear loss of the inner mitochondrial membrane potential with the extension of infection time. In contrast, cells with no virus infection or infected with EVA71 (UV) maintained high levels of TMRE fluorescence. As a positive control, cells treated with CCCP resulted in a near complete loss of their inner mitochondrial membrane potential (Fig. 1d).

**EVA71 induces the translocation and activation of Bax**

The above results indicated that EVA71 induces the loss of the inner mitochondrial membrane potential and the release of Cyt c from mitochondria. Therefore, we asked if EVA71 functioned by modulating members of the Bcl-2 family, which tightly regulate the mitochondria checkpoint in apoptotic cells. Since pro-apoptotic proteins Bax and Bak in their active forms are the main proteins responsible for the destabilization of the mitochondrial membrane, we first analysed the activation of Bak and Bax during EVA71 (BrCr) infection. Bak activation requires an initial conformational change, which is characterized by the exposure of the N-terminus. To determine if EVA71 interfered with the initial conformational change in Bak, HeLa cells were infected with EVA71 or treated with staurosporine. The conformation of Bak was monitored using a conformation-specific anti-Bak antibody that recognizes the exposed
N-terminus; thus, activated Bak can be detected by flow cytometry. Cells treated with staurosporine resulted in a clear increase in fluorescence intensity, indicating an increase in epitope availability for the conformation-specific antibody of Bak (Fig. 2a). In contrast, infection with EVA71 (BrCr) or EVA71 (UV) exhibited no clear induction of the N-terminal exposure of Bak. No increase in fluorescence intensity was detected in cells stained with an isotype control antibody.

To determine if EVA71 induced Bax translocation to the mitochondria and consequently resulted in Bax conformational change, we harvested and fractionated cells infected with EVA71. Mitochondria and cytosolic fractions were separated by centrifugation, and equal amounts of proteins were separated by SDS-PAGE followed by immunoblotting with anti-BaxNT antibody. A very weak signal that represents a small fraction of loosely attached inactive Bax on the mitochondria fraction was detected in mock- or EVA71

Fig. 1. EVA71 activates mitochondrial apoptotic pathway. (a) Detection of cell apoptosis that was induced by EVA71 infection. HeLa cells were infected with EVA71 strain HuBe09, strain AnHu09, strain BrCr and EVA71 (UV) at an m.o.i. of 2 p.f.u. cell$^{-1}$. Cells were then harvested at the indicated time, stained with annexin V-FITC and analysed by flow cytometry as described in the Methods. (b) The effects of caspase inhibitors on cell apoptosis induced by EVA71. Cells were either mock infected or infected with EVA71 strain BrCr or EVA71 (UV) at an m.o.i. of 2 p.f.u. cell$^{-1}$ following treatment with zDEVD.fmk and zLEHD.fmk or DMSO for 18 h. Cells were then harvested and stained with annexin V-FITC and analysed by flow cytometry. (c) EVA71 induces the release of Cyt c and the activation of caspase-3 and caspase-9. Cells were infected with EVA71 as described above. The cytoplasmic fractions were separated and immunoblotted with Cyt c, caspase-3 and caspase-9. WB, Western blot. (d) EVA71 induces the loss of the inner mitochondrial membrane potential. Cells were infected with EVA71 and stained with TMRE at the indicated time post infection. Mitochondrial membrane potential was then measured by flow cytometry as described in the Methods. Control, cells with no EVA71 infection.
(UV)-infected cells. In contrast, a significantly larger amount of activated Bax in the mitochondria fractions from EVA71-infected cells was observed (Fig. 2b). Accordingly, Bax in cytosolic fractions from EVA71 (BrCr)-infected cells was notably decreased, indicating that Bax translocates from the cytoplasm to the mitochondria caused by EVA71 (BrCr) infection. The cellular translocation of Bax was further determined by immunofluorescence staining with Bax antibody; mitochondria were stained with MitoTracker Red. A significant amount of dotted Bax immunofluorescence signal that co-localized with mitochondria was detected in cells with EVA71 (BrCr) infection or staurosporine treatment, whereas no co-localization of Bax and mitochondria was observed in mock- or EVA71 (UV)-infected cells, which further confirmed that Bax was translocated to the mitochondria in EVA71 (BrCr)-infected cells (Fig. 2c).

To investigate if EVA71 induces Bax or Bak homo-oligomerization, we monitored the appearance of higher molecular weight Bax or Bak complexes. Mitochondria were isolated from EVA71 (BrCr)-infected or mock-infected
cells, and Bax and Bak oligomerization was monitored by chemical cross-linking with bismaleimidoehexane (BMH) and assessed by Western blotting. In the absence of BMH, Bax and Bak showed approximately 20 and 26 kDa, respectively. However, upon the addition of BMH, Bax and Bak displayed a slower mobility as a result of intra-molecular cross-linking as observed in the staurosporine-treated cells used as positive control (Fig. 3). Results indicated that mitochondria purified from EVA71 (BrCr)-infected cells showed the absence of Bak homo-oligomers (Fig. 3a), in contrast to the appearance of the intra-molecular cross-linked Bak species and the formation of Bax oligomers indicative of Bax activation (Fig. 3b). Collectively, the above results indicated that EVA71 (BrCr) induced the homo-oligomerization of Bax but not Bak.

Apoptosis induced by EVA71 is Bax dependent in HeLa cells

To determine the importance of Bax in EVA71-induced mitochondrial apoptosis, we utilized small interfering RNA (siRNA) to knock down the expression of Bax and Bak. HeLa cells were transfected with siRNA against Bak, Bax or both Bak and Bax, caspase-8 or nontargeting siRNA as a control. At 48 h post transfection, cells were infected with EVA71, and cell lysate was prepared and analysed by SDS-PAGE and immunoblotted with anti-Bax, anti-Bak or caspase-8 antibodies. Results showed that the expression levels of Bax, Bak and caspase-8 were similarly reduced in siBak, siBax, siBax/Bak and siCaspase-8 cells, respectively, compared to that in nontargeting siRNA-transfected cells (Fig. 4a). Expression levels of Bak, Bak and caspase-8 were similarly low at all times post transfection (data not shown), while the total amount of Cyt c, Bcl-2, Bcl-XL, Bid, Bim, Bad or Mcl-1 was not affected by siRNA transfection. Next, to analyse the impact of Bak, Bax and caspase-8 knockdown on cell viability, cells were infected with EVA71 (BrCr) or EVA71 (UV) at 48 h post transfection for all subsequent experiments. The cell viability was determined 24 h post infection. Results showed that EVA71 (BrCr) rapidly induced the decrease of cell viability in the siBak, siCaspase-8 and nontargeting siRNA-transfected cells. However, the loss of cell viability induced by EVA71 infection was delayed in both Bax siRNA and Bak plus Bax siRNA-transfected cells, with less than 15% of the loss of cell viability (Fig. 4b).

The release of Cyt c from mitochondria is a direct consequence of mitochondrial outer membrane permeabilization, and we therefore confirmed the activation preferences described above by analysing Cyt c release from mitochondria. The presence of Cyt c in cytosolic fractions was detected by immunoblotting with anti-Cyt c antibody. Results showed that the supernatants of control siRNA and Bak siRNA-transfected cells contained higher amounts of Cyt c compared with that of the Bax siRNA and Bak plus Bax siRNA-transfected cells. Strikingly, the loss of Cyt c from mitochondria in Bax siRNA-transfected cells was significantly attenuated (Fig. 4c), indicating that the release of Cyt c from mitochondria to cytosol induced by EVA71 (BrCr) is Bax dependent in HeLa cells. The induction of apoptosis in EVA71 (BrCr)-infected cells was further confirmed by analysis of caspase-3-like activity in cell lysate. Cells treated with staurosporine were used as a positive control. Results showed that control siRNA-, caspase-8 siRNA- and Bak siRNA-transfected cells had higher levels of caspase-3-like activity, while the Bax siRNA- and Bak plus Bax siRNA-transfected cells had significantly less active caspase-3 than control siRNA-transfected cells (Fig. 4d). The effect of EVA71 (BrCr) on mitochondrial membrane potential was also analysed by flow cytometry at the indicated times post infection. Control siRNA-, caspase-8 siRNA- and Bak siRNA-transfected cells that were infected with EVA71 (BrCr) showed a clear loss of the inner mitochondrial membrane potential (Fig. 5). In contrast, Bax- and Bak plus Bax- siRNA-transfected cells infected with EVA71 maintained a high level of TMRE, indicating a very low level of inner mitochondrial membrane potential loss. As expected, control or EVA71 (UV)-infected cells maintained a high level of TMRE fluorescence. Collectively, the data in Figs 4 and 5 indicated that the silence of Bax expression inhibits the release of Cyt c from mitochondria and the rate of mitochondrial membrane potential loss induced by EVA71 (BrCr), while the silence of Bak or caspase-8 expression does not.
Isolated mitochondria from EVA71-infected cells induced the activation of Bax

BH3-only proteins, such as Bim or Bid, exert direct Bax-activating properties through their eponymous BH3 domain. However, no obvious activation of Bid and Bim was observed during EVA71 infection (Fig. 6a). This result led us to hypothesize that EVA71 (BrCr) would employ other mechanisms to modulate the mitochondria to recruit Bax. Thus, purified recombinant Bax protein was added to mitochondria that had been isolated from Bax siRNA cells infected with EVA71 or not. After co-incubation of mitochondria with either Bax or diluent control for 1 h, the mitochondria were pelleted by centrifugation, and the resulting supernatants were analysed for the presence of Cyt c by immunoblotting. Higher amounts of Cyt c were detected in the supernatants from Bax-treated mitochondria isolated from EVA71 (BrCr)-infected cells than from EVA71 (UV)- and mock-infected cells (Fig. 6b). Bax induced about 30% of the total Cyt c release from mitochondria under the conditions of these assays, based on comparisons with mitochondria treated by Triton X-100. Accordingly, Cyt c in mitochondria isolated from EVA71 (BrCr)-infected cells was notably decreased, indicating that Cyt c was released from the mitochondria to the supernatants. Recombinant Bax protein that associated with mitochondria was then analysed by immunoblotting with anti-His antibody. Results showed a low level of recombinant Bax in the mitochondria fractions from EVA71 (UV)- and mock-infected cells (Fig. 6b). In contrast, a significantly larger amount of recombinant Bax was detected in the mitochondria from EVA71 (BrCr)-infected cell diluents.
To investigate if Bax-treated mitochondria could release factors that induce the activation of DEVD-cleaving caspases, isolated mitochondria were treated in vitro for 1 h with either Bax protein or diluent control, followed by removal of the mitochondria by centrifugation and collection of the resulting supernatants. These supernatants and control supernatants were then added to cytosolic fractions derived from HeLa cells, and caspase activity was measured after 0.5 h by spectrofluorimetric assays with DEVD-AFC (7-amino-4-trifluoromethylcoumarin) as a substrate. The combination of Bax plus mitochondria derived from EVA71 (BrCr)-infected cells resulted in substantial elevations in caspase activity, whereas neither Bax plus mitochondria derived from EVA71 (UV)- nor mock-infected cells stimulated elevations in caspase activity. (Fig. 6c). Taken together, these results suggested that EVA71 recruited Bax to mitochondria by directly modulating cell mitochondria.

**Bcl-X<sub>L</sub> prevents EVA71-induced release of Cyt c from mitochondria**

Bcl-X<sub>L</sub> has been proven to undergo heterodimerization with Bax and inhibit its apoptotic effect. Thus, we examined the impact of Bcl-X<sub>L</sub> protein on EVA71-induced Bax activation and cell apoptosis [19]. A HeLa cell line that stably overexpressed Bcl-X<sub>L</sub>-HA (HeLa-Bcl-X<sub>L</sub>) was infected with EVA71. Twenty-four hours post infection, cell apoptosis was analysed by annexin V-FITC staining. HeLa-Bcl-X<sub>L</sub> cells infected with EVA71 displayed a very low annexin V fluorescence intensity, indicating that Bcl-X<sub>L</sub> could abrogate the apoptosis induced by EVA71 infection. To further confirm the above results, we investigated the release of Cyt c from mitochondria in HeLa-Bcl-X<sub>L</sub> cells. Results indicated that the overexpression of Bcl-X<sub>L</sub> completely inhibited the EVA71-induced Cyt c release. A band corresponding to Cyt c was detected in HeLa but not in HeLa-Bcl-X<sub>L</sub> cells infected with EVA71 (Fig. 7b). The caspase-3-like activity in HeLa and HeLa-Bcl-X<sub>L</sub> cells was also determined. In contrast to the dramatic increase seen in staurosporine-treated or EVA71-infected HeLa cells, a relatively modest change of activated caspase-3 in HeLa-Bcl-X<sub>L</sub> cells infected with EVA71 was detected (Fig. 7c).
addition, the loss of the inner mitochondrial membrane potential was also analysed in HeLa-Bcl-XL cells that were infected with EVA71. HeLa cells with EVA71 infection or treated with staurosporine showed a significant loss of the inner mitochondrial membrane potential, while HeLa-Bcl-XL cells infected with EVA71 maintained high levels of TMRE fluorescence, demonstrating that Bcl-XL overexpression inhibited the loss of the inner mitochondrial membrane potential during EVA71 infection (Fig. 7d).

Mitochondria fractions were then separated by centrifugation, and equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-BaxNT antibody. A significantly larger amount of Bax in the mitochondria fractions in HeLa-Bcl-XL cells that were infected with EVA71 was notably decreased. This suggested that Bax translocation from the cytoplasm to mitochondria in HeLa-Bcl-XL cells with EVA71 infection was inhibited by Bcl-XL expression. HeLa-Bcl-XL or HeLa cells that were infected or mock infected with EVA71 were collected and lysed in 2 % CHAPS buffer followed by immunoprecipitation with anti-Bax6A7 antibody as described above. A large amount of active Bax with conformational change was detected in staurosporine-treated or EVA71-infected HeLa cells. No active Bax was observed in HeLa-Bcl-XL cells infected with EVA71 (Fig. 7f). Collectively, the above results indicated that overexpression of the anti-apoptotic protein Bcl-XL caused a dramatic reduction in apoptotic rate of cells infected with EVA71.

Fig. 6. Bax induces Cyt c release from isolated mitochondria with EVA71 infection. (a) Western blot analysis of the activation of Bid and Bim during EVA71 infection. Cells were infected with EVA71 (BrCr) at an m.o.i. of 2 p.f.u. cell−1. Twelve hours post infection, cell lysates were prepared and analysed by SDS-PAGE and immunoblotted with specific antibodies to Bid and Bim. TPA, cells treated with 200 nM tetradecylphosphonic acid for 30 min. Etoposide, cells treated with 25 µM etoposide for 30 min. (b) Western blot analysis of the effect of recombinant Bax on Cyt c release in isolated mitochondria from siBax HeLa cells that were infected with EVA71. Mitochondria were incubated for 2 h with 1 or 0.5 µM recombinant Bax, Triton (0.2 %, v/v), BSA (1 µM) or neither of these reagents (control) at room temperature. Mitochondria were then pelleted by centrifugation, and the resulting supernatants were immunoblotted with antibody to Cyt c. The pellet fractions were lysed and immunoblotted with antibody to His tag or Cyt c. (c) Bax induces the release of mitochondria factors that trigger processing and activation of cytosolic caspase-3 in mitochondria with EVA71 infection. Supernatants from mitochondria that had been incubated with Bax, Triton (0.2 %, v/v), BSA (1 µM) or none of these reagents were added to the purified cytosol. Caspase-3 activity was measured as described in the Methods.
To further evaluate the role of Bcl-X\textsubscript{L} in EVA71-induced apoptosis, we investigated the effect of purified recombinant Bcl-X\textsubscript{L} protein on Bax-induced release of Cyt c from mitochondria in vitro. Mitochondria that were purified from siBax-HeLa cells infected or mock infected with EVA71 were incubated with recombinant Bcl-X\textsubscript{L} and/or protein Bax. Mitochondria were then pelleted, and the supernatants were analysed by immunoblotting with Cyt c antibody. Bax-induced Cyt c release from mitochondria was almost completely inhibited by the addition of Bcl-X\textsubscript{L}. No Cyt c release was observed in the supernatants from Bcl-X\textsubscript{L} or Bcl-X\textsubscript{L} plus Bax-treated mitochondria (Fig. 8a). Supernatants were also used for caspase-3 activity assays; results showed that recombinant Bcl-X\textsubscript{L} protein also suppressed the proteolytic processing of caspase-3 that was induced by co-addition of Bax protein and mitochondria to HeLa cytosolic extracts. In contrast, when Cyt c addition exogenously was used to induce processing of caspase-3 in HeLa cells infected with EVA71 (m.o.i., 0.5) for 24 h and stained by annexin V-FITC. Staurosporine, cells treated with staurosporine for 12 h. (b) Western blot analysis of the Cyt c in the cytosolic fraction of HeLa or HeLa-Bcl-X\textsubscript{L} (a HeLa cell line that stably expresses Bcl-X\textsubscript{L}–HA) cells that were infected with EVA71. Stau., cells treated with staurosporine (30 nM) for 12 h. (c) Analysis of the caspase-3-like activity in cell lysates of HeLa-Bcl-X\textsubscript{L} or HeLa cells infected with EVA71 or EVA71 (UV). Caspase-3 activity was determined as described in the Methods. (d) Flow cytometry analysis of the inner mitochondrial membrane potential in HeLa-Bcl-X\textsubscript{L} cells with EVA71 infection. HeLa or HeLa-Bcl-X\textsubscript{L} cells were infected with EVA71 for 12 h; cells were then stained with TMRE for 10 min at 37°C and subjected to flow cytometry analysis. Control, cells with no EVA71 infection. Staurosporine, cells treated with 30 nM staurosporine. (e) Western blot analysis of the Bax in mitochondria fractions of HeLa-Bcl-X\textsubscript{L} or HeLa cells infected or mock infected with EVA71. Cells were infected or mock infected with EVA71 for 12 h. Mitochondria fractions were then separated, and equal amounts of proteins were immunoblotted with anti-BaxNT or anti-Cox IV (internal control of mitochondria fraction). (f) Western blot analysis of the activated Bax in HeLa-Bcl-X\textsubscript{L} or HeLa cells infected or mock infected with EVA71. Cells were lysed and immunoprecipitated with anti-Bax6A7 antibody. Equal amounts of the precipitated proteins and cell lysates were immunoblotted with antibody to Bax. Stau., cells treated with staurosporine (30 nM) for 12 h.
cytosolic extracts, recombinant Bcl-X_L protein failed to function, suggesting that Bcl-X_L could not provide protection once Cyt c had been released into the cytosol (Fig. 8b).

DISCUSSION

As one of the main pathogenic agents that cause hand, foot and mouth disease in young children, EVA71 infection usually induces cell apoptosis resulting from tissue damage, which is possibly the process of pathogenesis [20, 21]. EVA71 infection induces classic signs of apoptosis in all detected cells, including the efflux of Cyt c from mitochondria, and subsequent cleavage of caspase-9 and caspase-3 [22]. The mitochondrial pathway of apoptosis mediated by activation and cleavage of caspase-9 is a main pathway in EVA71-induced apoptosis. However, the precise signal pathways leading to apoptosis during EVA71 infection have remained unclear. In this study, we provided the evidence that EVA71-induced apoptosis relies on Bax activation and translocation. Mitochondria from EVA71-infected cells recruited Bax and induced Bax activation. This is a new insight into understanding the interplay between apoptosis and the EVA71 infection.

Apoptosis is one of the common cellular responses to many viral infections. At the early stage of viral infection, cell apoptosis may function as a defence reaction to prevent generation and spread of the viral progeny. On the other hand, to enable efficient viral production, many viruses trigger apoptosis at the late stage of infection and spread of progeny to neighbouring cells. In the family Picornaviridae, several viruses have been demonstrated to induce cell death by apoptosis [23, 24]. However, for these viruses, relatively little is known about the mechanism involved. It has been shown that coxsackievirus B3 induces apoptosis through caspase activation [25, 26]. A coxsackievirus B2 mutant strain with a single amino acid change (glutamine to lysine at position 164) induces apoptosis including caspase activation and DNA degradation in rhabdomyosarcoma cells [27]. Avian encephalomyelitis virus localizes to mitochondria and promotes Cyt c efflux into the cytosol, which in turn activates upstream caspase-9 and downstream caspase-3, leading to apoptosis [28]. It is reported that the activation of the apoptotic mitochondrial pathway in PV-infected neuronal IMR5 cells is Bax dependent [29]. As a member of human enterovirus species A of the genus Enterovirus within the family Picornaviridae, EVA71 infection has been proved to induce the activation of the mitochondrial pathway of apoptosis [30]. This is consistent with our results that EVA71 induced the release of Cyt c from mitochondria and the subsequent caspase-9 and caspase-3 activation. Cells that lack caspase-8 expression also displayed a high level of Cyt c release from mitochondria and the loss of the inner mitochondrial membrane potential, suggesting that apoptosis induced by EVA71 may not depend on caspase-8 activation.

In the absence of an apoptotic trigger, the majority of Bax is found in cytoplasm or loosely associated with mitochondrial membrane. Following an apoptotic trigger, Bax exposes the N-terminus and liberates the C-terminal TM domain, resulting in mitochondrial membrane insertion, homo-oligomerization of Bax and the release of Cyt c. As a pro-apoptotic member of the Bcl-2 family, how Bax induces these mitochondria alterations is currently controversial. It is possible that Bax creates pores in the outer membrane that are large enough to allow the escape of Cyt c. Alternatively, Bax might indirectly alter the permeability of the outer membrane through interactions with other proteins. Of the pro-apoptotic BH3-only proteins, Bim and Bid have been shown to directly activate Bax and Bak with a particularly high potency and are consequently termed ‘activator’ BH3-only proteins. Bim and Bid also bind and inactivate the major anti-apoptotic proteins including Bcl-2 and Bcl-X_L. However, in our study, no obvious activation of Bid and Bim was observed during EVA71 infection, indicating that the activation of Bax by EVA71 was not a consequence of the cleavage of Bid by caspase-8 or the activation of Bim.

Viral proteins often induce or antagonize the effects of cellular pro-apoptotic Bcl-2 family proteins, especially Bax and Bak. For example, Epstein–Barr virus codes for BALF1, a Bcl-2 homologue which localizes to mitochondria, interacts with pro-apoptotic Bax and Bak and induces cell apoptosis [31]. M11L, encoded by myxoma virus, E1B19K, encoded by adenovirus, F1L and E1B-19K, encoded by vaccinia virus, and ORF125, encoded by poxvirus, inactivate Bak and/or Bax to inhibit apoptosis [32–34]. In the present study, a
great number of recombinant Bax proteins were detected to bind and undergo oligomers in isolated mitochondria from cells that were infected with EVA71, indicating that EVA71 infection could recruit Bax to mitochondria by directly modulating the conformational activation of Bax. This result leads us to hypothesize that protein(s) encoded by EVA71 may localize to mitochondria and recruit Bax. The interaction of Bax with protein(s) encoded by EVA71 triggers the exposure of Bax TM domain, resulting in mitochondrial membrane insertion and Cyt c release. Otherwise, EVA71 may create a mitochondria environment that is favourable for binding or oligomerization of Bax and other molecules from mitochondria. EVA71 may also modulate Bax activity by sequestering Bax ‘inhibitor’ molecules. Further experiments are needed to better delineate the mechanism(s) by which the hypothesized protein(s) encoded by EVA71 that may localize to mitochondria recruit Bax and activate Bax.

Virus-induced apoptosis has been shown to be an important component of PV-induced cell death and tissue injury in the central nervous system of infected mice [35, 36]. EVA71 infections are usually accompanied by severe neurological complications such as aseptic meningitis, acute flaccid paralysis, encephalitis and other rarer manifestations [37, 38]. These neurological complications can sometimes be fatal, and neurogenic pulmonary oedema is thought to be the main disease process in fatal cases. We could hypothesize that EVA71-induced apoptosis may play an important role in the pathogenesis of disease in the central nervous system, since apoptosis in nonrenewable cells may result in an irreversible pathology. Therefore, this work represents an attempt to unveil the relevance of EVA71-induced apoptosis for human diseases.

METHODS

Cell culture and virus isolation

The prototype EVA71 BrCr strain was a gift from Qi Jin (Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Beijing, People’s Republic of China). The prototype EVA71 clinical strains Hunan 09 and HeN09 were gifts from Zhang Bo (Wuhan Institute of Virology, Chinese Academy Of Sciences, Wuhan, People’s Republic of China). The EVA71 (UV) was a mutation of EVA71 (BrCr) treated by ultraviolet. The human cervical (HeLa) cells were propagated and maintained in Dulbecco’s modified Eagle’s medium supplemented with antibiotics (penicillin and streptomycin) and 10% foetal bovine serum (Invitrogen) at 37°C in the presence of 5% CO2. To generate a HeLa cell line that stably overexpresses Bcl-XL-HA (HeLa-Bcl-XL), the full-length human cDNA for the Bcl-XL was cloned into the restriction site of the pcDNA3.0-HA plasmid, after digesting with EcoRI/NotI. The recombinant plasmid was then transfected into HeLa cells by using the Lipofectamine 3000 transfection reagent. The expression of Bcl-XL-HA in stable transfecants was confirmed by Western blot analysis.

Virus infection was performed as described before [30]. In short, semi-confluent monolayers of HeLa cells were infected with EVA71. After adsorption for 30 min at 37°C, the cells were washed twice with PBS buffer and overlaid with double modified Eagle’s medium containing 10% calf serum. Cells were cultured at 37°C in the presence of 5% CO2.

The human Bax was expressed recombinantly and prepared as described [39]. Proteins (0.2–0.4 mg ml−1) were dialysed into 20 mM HEPES (pH 7.5), 10 mM KCl, 20 mM MgCl2 and 1 mM EDTA before use in all experiments.

Reagents and antibodies

The protease inhibitor cocktail, staurosporine, CCCP, TMRE, MitoTracker Red, tetradecylyphosphonic acid and etoposide were obtained from Sigma-Aldrich. DMSO, BMH and Lipofectamine 3000 transfection reagent were obtained from Invitrogen.

Rabbit anti-Bak antibody, rabbit monoclonal antibodies against Bax6A7 (specifically recognizing activated Bax protein with an exposed N-terminus), BaxNT (recognizing both native and activated Bax), Bid, Bim, Cyt c, Bcl-2, Bcl-XL, caspase-3, caspase-8, caspase-9, Cox IV, TSPO (mitochondrial outer membrane translocator protein) and Mcl-1 were obtained from Epitomics. Mouse anti-GFP, mouse anti-His and mouse anti-Flag monoclonal antibodies and FITC-conjugated anti-rabbit IgG antibody used in immunofluorescence studies and Western blot analyses were all obtained from Santa Cruz Biotechnology. Annexin V-FITC was obtained from Biyotime.

Cell viability analysis, apoptosis assay and measurement of mitochondrial membrane potential

The cell viability was analysed as described before [40]. To analyse cell apoptosis, cells were harvested and resuspended in annexin V binding buffer at a concentration of 1x10⁶ cells ml⁻¹. Cells were mixed with annexin V-FITC and then incubated for 15 min at room temperature in the dark. The fluorescence signals were detected by BD FACSCalibur (Becton Dickinson), and data were analysed with CellQuest software (Becton Dickinson).

TMRE (Molecular Probes) was used to measure the changes of mitochondrial membrane potential in cells according to the manufacturer’s instructions. In short, cells were stained by incubating them in medium containing 0.2 μM TMRE for 20 min at 37°C. The fluorescence signals were then detected by flow cytometry.

Mitochondria purification, detection of Cyt c and Bak and Bax cross-linking

Mitochondria, recombinant human Bax and Bcl-XL were prepared as described before [41]. To monitor Cyt c release, purified mitochondria were incubated with recombinant protein Bax with or without Bcl-XL for 30 min at 25°C. The mitochondria were pelleted by centrifugation at 8000 g for
10 min, solubilized in RIPA buffer and analysed by immunoblotting with the indicated antibodies.

For Bax and Bak cross-linking, pelleted mitochondria were pelleted and resuspended in the conjugation buffer, PBS (pH 7.2). Mitochondria were then subjected to cross-linking using 0.2 mM BMH for 2 h at 4 °C. Cross-linking was quenched in a quenching solution at a final concentration of 30 mM. Protein samples were then separated by SDS-PAGE and analysed by Western blotting with anti-Bax or anti-Bak antibody.

**siRNAs and transient transfection**

siRNAs that target human Bak (5’-GGAGCUGCAGAG-GAUGAUUTT-3’), Bax (5’-GGUCACCUAACCUUGC-CAATT-3’) and siCaspase-8 (5’-AACCUCGGGGAUAC UGUCUGA-3’), and nontargeted (siControl) siRNAs (5’-UUUCUGAAGCUGUCAGUTT-3’) were chemically synthesized by Guangzhou RiboBio. For transient transfection, Lipofectamine 3000 (Invitrogen) was used according to the manufacturer. In short, HeLa cells were plated in culture dishes and allowed to grow for 24 h to 90 % confluency. A mixture of Opti-MEM medium and Lipofectamine 3000 was incubated for 5 min at room temperature and then incubated with siRNA (50 nM) or recombinant plasmid (2 µg/ml) for 10 min at room temperature. This mixture including siRNA or recombinant plasmid was then added to the well. Twenty-four hours after transfection, the medium was changed, and analyses were performed at the indicated time after transfection. Gene silencing or protein expression was verified by detecting proteins by Western blot analysis after transient transfection of HeLa cells with siRNA.

**Confocal microscopy, immunoprecipitation, cell protein extraction and Western blot**

For confocal microscopy, cells were grown on coverslips and infected with EVA71. Cell mitochondria were stained using MitoTracker Red (50 nM) according to the manufacturer. The stained cells were then fixed in paraformaldehyde in phosphate buffer (pH 7.4) for 30 min. After three washes with PBS, the cells were examined under an inverted fluorescence or a confocal scanning microscope using an excitation wavelengths of 550 and 490 nm.

For immunoprecipitation, cell lysates were preimmunoprecipitated with protein A/G agarose beads. After a short centrifugation, the precipitates were incubated with either mouse IgG-conjugated agarose beads or anti-Bax6A7-conjugated agarose beads. After 2 h incubation at 4 °C, the beads were washed with lysis buffer and heat denatured in sample loading buffer (50 mM Tris/HCl (pH 6.8), 100 mM dithiothreitol, 2 % SDS, 0.1 % bromophenol blue and 10 % glycerol). After a brief centrifugation, the proteins in supernatants were separated by SDS-PAGE followed by Western blot analysis.

Cell protein extraction and Western blot were performed as described before [30].

**Assay for and activation of mitochondria-dependent caspase-3**

Cytosolic extracts were prepared from HeLa cells as described before [41]. Supernatants (2 ml) from Bax-treated or control mitochondria were incubated with 0.5 ml of the cytosol extract at 25 °C for 0.5 h. Aliquots were utilized for caspase-3 assay according to the instructions of the manufacturer (Santa Cruz Biototechnology).

**Statistical analysis**

Data were subjected to one-way ANOVA with factors of treatment and expressed as means±SD. Comparisons between any two groups were performed by unpaired Student’s t-tests: * Indicates a significant difference at P values of 0.02 compared with the control; ** indicates a significant difference at P values of 0.05 compared with the control.

**Funding information**

This work is supported by a grant from the National Natural Science Foundation of China (grant no. 31270211).

**Acknowledgements**

We are grateful to Dr Yangguang Li, of the Department of Medicine, School of Medicine and Public Health, University of Wisconsin-Madison, for his help during the preparation of the manuscript. We also thank Professor Ruigang Wang, College of Life Sciences, Inner Mongolia Agriculture University, for technical help with the performance of confocal microscopy.

**Conflicts of interest**

The authors declare that they have no conflict of interest.

**Ethical statement**

This article does not contain any studies with human participants or animals performed by any of the authors.

**References**


Downloaded from www.microbiologyresearch.org by IP: 54.70.40.11
On: Mon, 17 Jun 2019 12:52:44


