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Short communication

Intrinsic, extrinsic and endoplasmic reticulum stress-induced apoptosis in RK13 cells infected with equine arteritis virus

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ABSTRACT

The modulation of the expression of caspases by viruses influences the cell survival of different cell types. Equine arteritis virus (EAV) induces apoptosis of BHK21 and Vero cell lines, but it is not known whether EAV induces apoptosis in RK13 cells, a common cell line routinely used in EAV diagnosis and research. In this study, we determined that caspase-3 expression was triggered after infection of RK13 cells with EAV in a time- and dose-dependent manner. We also detected caspase-8 and caspase-9 activation, indicating the stimulation of both extrinsic and intrinsic apoptosis pathways. Finally, we found caspase-12 activation, an indicator of endoplasmic reticulum stress-induced apoptosis. The variability observed in the apoptotic response in the different cell lines demonstrates that apoptosis depends on the distinctive sensitivity of each cell line used for investigation.

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The order Nidovirales comprises the families Arteriviridae, Coronaviridae, Mesoniviridae, and Roniviridae (de Groot et al., 2012; Lauber et al., 2012). Equine arteritis virus (EAV) has been classified as a member of the family Arteriviridae and grouped together with porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV) (Snijder et al., 2013). It has been previously shown that EAV induces apoptosis *in vitro* (Archambault and St-Laurent, 2000), DNA fragmentation and caspase activation (Cholleti et al., 2013; St-Louis and Archambault, 2007).

Caspases play a central role in apoptosis and their activation is a highly regulated mechanism. While the activation of caspase-9 is related to the induction of the intrinsic apoptotic pathway through cytochrome-C release from mitochondria (Neumann et al., 2015), the activation of caspase-8 is linked to the extrinsic apoptotic pathway (Elmore, 2007). Although, endoplasmic reticulum (ER) stress-induced apoptosis is still poorly understood, caspase-12 has been proposed as a key mediator of this mechanism (Szegezdi et al., 2003).

Viruses have evolved multiple strategies to control the apoptotic response. Some viruses have developed strategies to maximize apoptosis, facilitating virus spread, whereas others have evolved specific mechanisms to inhibit apoptosis, establishing and maintaining virus persistence (Nakamura-López et al., 2011Roulston et al., 1999). Also, several viral proteins have been described to modulate apoptosis either by activation (Metz et al., 2014) or inhibition (Lee et al., 2005).

Induction of apoptosis by EAV was first shown in cultured African green monkey kidney cells (Vero cells) (Archambault and St Laurent, 2000) and the activation of caspase-8 and caspase-9 was later demonstrated in this cell line, by using the EAV T1329 Canadian isolate (St-Louis and Archambault, 2007). However, two other EAV strains, SP3A and Arvac, activate only the intrinsic apoptosis pathway (caspase-9) in Baby Hamster Kidney (BHK21) cells (Cholleti et al., 2013). Although apoptosis is a widely conserved mechanism in mammalian cells, these findings evidence differences in apoptosis induction in different EAV strains and cell lines that could be relevant both during *in vivo* infection and in laboratory studies.

Given that previous EAV-induced apoptosis studies have been performed in Vero and BHK21 cells, in the present study we decided to evaluate caspase activation in the Rabbit Kidney 13 (RK13) cell line to gain an insight into the caspase activation pathways in this

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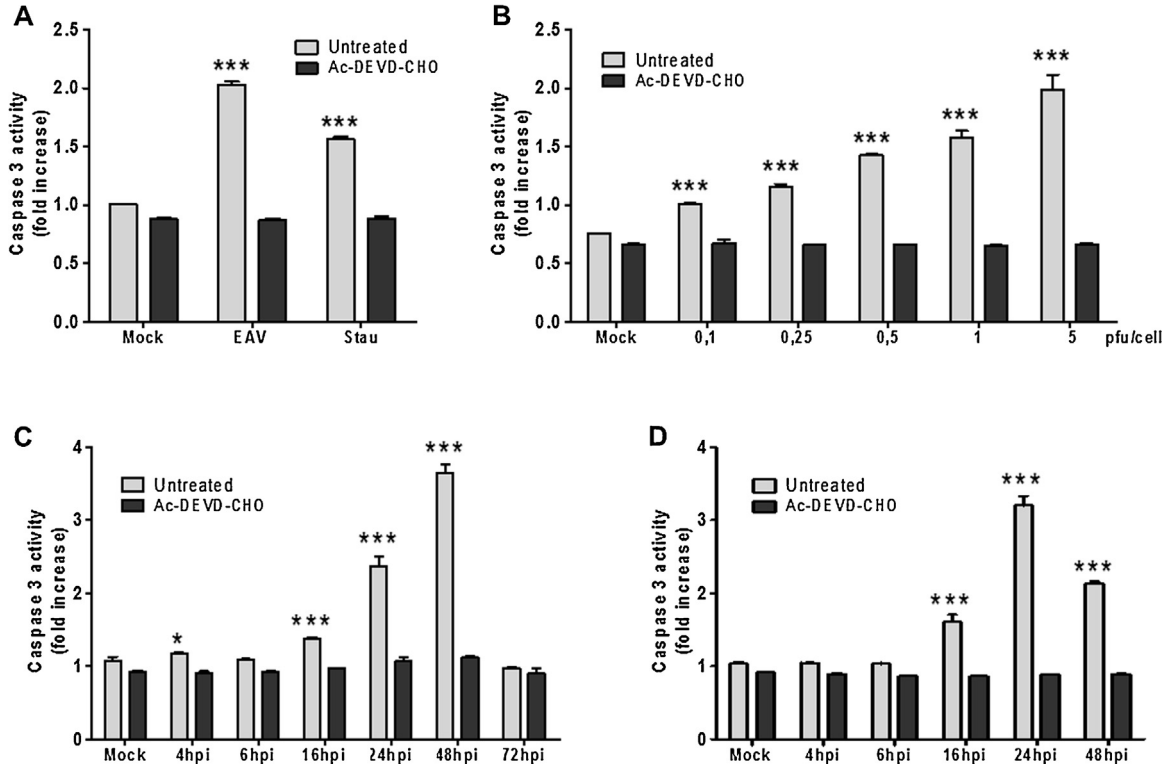

Fig. 1. Caspase-3 activation after equine arteritis virus (EAV) Bucyrus strain infection of RK13 cells (white bars) at a multiplicity of infection (MOI) of 1 pfu/cell in (1A) or at several MOIs: 0.1, 0.25, 0.5, 1 and 5 pfu/cell respectively at 24 hpi (1B) or cells infected at a MOI of 0.25 (1C) or 1 pfu/cell (1D) collected at several times post-infection, as indicated (4, 6, 16 24, 48 and 72 hpi). Also, infected RK13 cells were treated with an inhibitor of caspase-3, Ac-DEVD-CHO (black bars). Staurosporine-treated RK13 cells and mock-infected cells were used as positive and negative controls, respectively. Data from fluorometric assays in triplicates were normalized to control values of mock-infected cells at 48 hpi and represented in graphs as mean and standard deviation of caspase activity fold-increase. Statistically significant differences are indicated by asterisks: ($^{***}p < 0.001$, $^{**}p < 0.01$; $^{*}p < 0.05$).

cell line as it is routinely used in diagnostic tests such as viral neutralization as well as in EAV isolation assays.

In our experiments, we used EAV Bucyrus strain (Pubmed Reference: DQ846750) kindly provided by M. Agüero from Laboratorio Central de Veterinaria (LCV), Algete, Madrid, Spain. The virus titer was 1.35×10^7 pfu/ml measured by end-point dilution assay (Scotti, 1977).

As there was no information available about caspase activation in EAV-infected RK13 cells, we started evaluating caspase-3 activity. For this purpose, a freshly confluent RK13 monolayer was seeded on a six-well plate and infected with EAV at a multiplicity of infection (MOI) of 1 pfu/cell. This condition was used as the starting-point for the study for the different caspases analyzed in this work, but other MOIs were assayed as described below. Staurosporine is a protein kinase inhibitor that induces apoptosis by activating caspase-3. Hence, staurosporine-treated RK13 cells and mock-infected cells were used as positive and negative controls respectively. Caspase activity values at any time point were relativized to negative control mock-infected cells at the time of maximal background apoptosis (48 hpi). Infected RK13 cells were treated with a specific caspase-3 inhibitor (Ac-DEVD-CHO) as an internal control of the assay. Cells were collected at twenty-four hour post-infection (hpi), except where otherwise indicated. Caspase-3 activation was analyzed using a caspase-3 fluorometric assay (Sigma) according to the manufacturer's instructions. This assay is based in the hydrolysis of the substrate acetyl-Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC), upon caspase-3 activation. After 60 min of reaction, AMC fluorescence (460 nm) was measured using a Tecan GENios fluorometer and XFlour 4 software (Tecan Switzerland). The concentration of AMC released was calculated against a calibration curve standard. The experimental data were analyzed by one-way ANOVA by GraphPad Prism 5 software. For multiple comparisons, Bonferroni's correction was applied. A p value <0.05 was considered statistically significant as indicated in the figures. All the results are presented as the mean ± standard deviation (SD) from at least three independent experiments.

In the conditions assayed, we found caspase-3 activation after EAV infection, counted as relative fluorescence units and normalized to mock-infected cell values, which is represented in graphs as caspase activity fold-increase (Fig. 1A).

Once activation of caspase-3 was confirmed in RK13 cells at basal MOI conditions, we analyzed the effect of several virus concentrations using a range of MOIs from 0.1 to 5 (0.1, 0.25, 0.5, 1 and 5 pfu/cell). At 24 hpi, caspase-3 increased as the MOI increased (Fig. 1B). Nevertheless, an increase in both magnitudes (MOI and caspase-3 activation) did not follow a linear correlation. For example, a 50-fold increase of the MOI (0.1 and 5) correlated with an increase of less than 2-fold in caspase-3 activity.

To analyze the profile of caspase-3 activation along time, we performed caspase activity assays at a range of times after infection at 4, 6, 16, 24, 48 and 72 hpi at a MOI of 0.25 pfu/cell. Caspase-3 was activated at 24 hpi and peaked at 48 hpi (Fig. 1C), but was no longer detected at 72 hpi. At this time point, we observed detached and refringent cells, which indicated lysis of EAV-infected cells and probably a concomitant release of caspase-3 to the culture medium.

At a higher MOI (1 pfu/cell), the caspase-3 activation peak was observable at 24 hpi, *i.e.*, earlier than before, as it could be expected according to the higher viral titer used in the experiment (Fig. 1D). Again, the decline in caspase-3 yields at 48 hpi can be explained by the virus-induced cell lysis and subsequent caspase-3 release to the

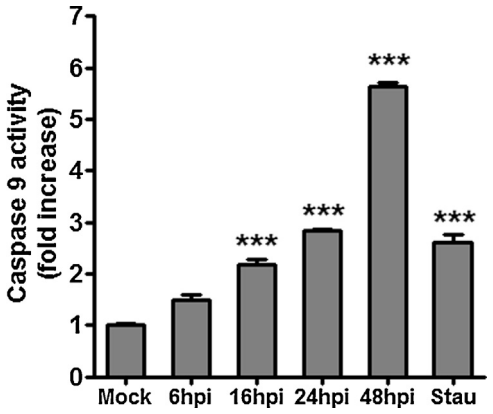

Fig. 2. Kinetics of caspase-9 activation in RK13 cells infected with EAV Bucyrus at a MOI of 0.25 pfu/cell and collected at several times post-infection (6, 16, 24 and 48 hpi). Staurosporine was included as a positive control. Caspase activities from triplicates were normalized to the corresponding values for mock-infected cells at 48 hpi.

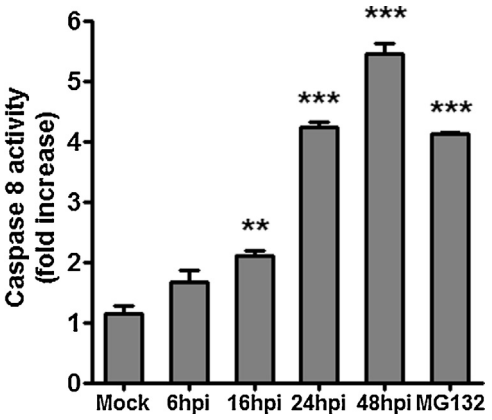

Fig. 3. Kinetics of caspase-8 activation in RK13 cells infected with EAV Bucyrus at a MOI of 0.25 pfu/cell and collected at several times post-infection (6, 16, 24 and 48 hpi). MG132 was used as positive control. Graphs represent caspase activity values from triplicates relative to control cells at 48 hpi.

culture medium. These results show that EAV induced an activation of caspase-3 that was dependent on the virus concentration used in the experiments and variable depending on the time after infection in which it was analyzed in infected RK13 cells.

EAV has been previously associated with the induction of caspase-9 activity in Vero (St-Louis and Archambault, 2007) and BHK21 cultured cells (Cholleti et al., 2013). To determine caspase-9 activation in RK13 cells, we used a caspase-9 fluorometric assay (BioVision) that detects the cleavage of the caspase recognition sequence -LEDH- bond to the fluorophore 7-amino-4-trifluoromethyl coumarin (AFC). The conditions assayed were similar to those in previous experiments. Cells were infected with EAV at a MOI of 0.25 pfu/cell and cells were collected at a range of times postinfection 6, 16, 24 and 48 hpi for caspase assays. The time point of 72 hpi was not used in subsequent assays given that the existing cells were lysed at these late times. We also observed that EAV induced caspase-9 activation in RK13 cells. Caspase-9 was detectable starting at 16 hpi and increased along infection compared with uninfected cells, with the highest level of activation found at 48 hpi (Fig. 2). In all experiments, caspase activation values were normalized to negative control values of mock-infected cells at 48 hpi.

We analyzed caspase-8 kinetics using caspase-8 fluorometric assay (BioVision) at a MOI of 0.25 pfu/cell at several time points. This assay detects the activity of caspase 8/FLICE which recognizes the sequence -IETD- bond to AFC. Upon cleavage of the substrate by caspase-8/FLICE, release of AFC emits fluorescence at 505 nm which was quantified using a fluorometer as above described. MG132 is a proteasome inhibitor that induces caspase-8-dependent apoptosis; then, cells treated with MG132 (Calbiochem) were used as positive controls of caspase-8 activation. Caspase activation values were normalized to negative control values of mock-infected cells at 48 hpi. This assay showed a four-fold activation at 24 hpi compared with the control, and a one-fold activation at 48 hpi with respect to the previous time point (24 hpi) (Fig. 3).

Previous studies have shown the activation of the apoptotic extrinsic mechanism (activation of caspase-8) in Vero cells using the EAV T1329 strain (St-Louis and Archambault, 2007). However, no previous studies have found evidence of caspase-8 activation in BHK21 cells using the EAV SP3A or Arvac strain, suggesting an influence of the virus strain or a distinct regulatory mechanism to undergo apoptosis in this cell line (Cholleti et al., 2013).

ER stress response is frequently initiated by viral infection. The production of large amounts of viral proteins overloads the ER and unfolded proteins accumulate and trigger stress (Wang et al., 2014; Waris et al., 2002). This entails other homeostatic responses, including ER-associated protein degradation (ERAD), autophagy and mitochondrial oxidative response, which ultimately lead to apoptosis. Hence, besides the intrinsic and extrinsic pathways, a third apoptotic pathway that induces multiple downstream apoptotic target genes such as caspase-12, caspase-7 (Nakagawa and Yuan, 2000; Rao et al., 2001) and proapoptotic c-Jun NH2-terminal kinase (JNK) can be elicited by prolonged ER stress (Li et al., 2006). The regulation and maintenance of ER homeostasis is highly relevant, given the several functions that take place in the lumen of the ER. The early replication and transcription complex of EAV is associated with double-membrane vesicles (DMVs), which most probably originate from the ER (Knoops et al., 2012). The ER stress response culminates in apoptosis, and caspase-12 has been proposed to be an initiator caspase involved in ER stress-induced apoptosis (Lamkanfi et al., 2004).

Taking into account the replication mechanism of EAV, we next analyzed the possible activation of caspase-12 using a caspase-12 fluorometric assay (BioVision). Again, this assay detects the caspase activation by the specific enzymatic activity on the sequence ATAD-AFC. The emission of released AFC was quantified using a fluorometer. Tunicamycin is an *N*-glycosylation inhibitor that induces ER stress, unfolded protein response and caspase-12 activation. Then, tunicamycin was used as a positive control of caspase-12 activation. Again, caspase activation values were normalized to negative control values of mock-infected cells at 48 hpi.

We detected caspase-12 activation upon EAV infection and its activation profile at several time points is shown in Fig. 4. A single activation peak was found at 24 hpi. This early apoptosis activation could be related to the early replication events in the DMVs in the process of mature EAV formation (Van der Meer et al., 1998). Also, infection of Madin-Darby bovine kidney (MDBK) cells with bovine viral diarrhea virus induces caspase-12 expression at 25 hpi, a fact that has been correlated with the onset of apoptosis (Jordan et al., 2002).

EAV and PRRSV infection have been previously associated with the activation of JNK and p38 MAPK signaling in BHK21 cells (Cholleti et al., 2013; Yin et al., 2012), linked to ER-induced apoptosis. Although caspase-12 activation has been reported for other viral infections (Galindo et al., 2012; Roberson et al., 2012; Wang et al., 2010), this is the first report of caspase-12 activation caused by an Arterivirus.

Caspase-12 has been shown to activate the intrinsic pathway both indirectly by the activation of mitochondria and the release of cytochrome-c and directly by cleaving procaspase-9

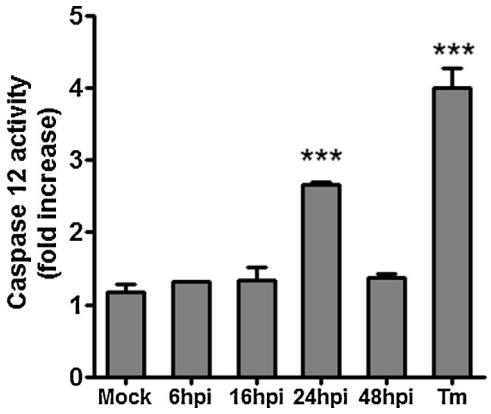

Fig. 4. Kinetics of caspase-12 activation in RK13 cells infected with EAV Bucyrus strain at a MOI of 0.25 pfu/cell and collected at several times post-infection (6, 16, 24 and 48 hpi). Tunicamycin was included as a positive control of caspase 12 activation. Caspase activity values from triplicates were normalized to mock-infected controls at 48 hpi and depicted in graphs.

(Morishima et al., 2002; Rao et al., 2002). Our results detected a two-fold increase in caspase-9 at 48 hpi, which could be associated with the previous activation of caspase-12 found at 24 hpi. Then, the results suggest that procaspase-9 could be a direct substrate of caspase-12, indicating a possible cross-talk between ER and the intrinsic apoptosis pathway. However, further studies should be carried out to support this possibility.

Previous studies of apoptosis in EAV have been inconclusive (Cholleti et al., 2013; St-Louis and Archambault, 2007). GP5 protein from EAV has been recently associated with the induction of apoptosis in insect cells (Metz et al., 2014). Other members of the family Arteriviridae, such as PRRSV, have been shown to induce apoptosis and there is evidence that PRRSV GP5 induces apoptosis (Gagnon et al., 2003; Suarez et al., 1996). We are thus conducting studies to investigate whether the EAV protein that is homolog to PRRSV GP5 is the protein responsible for apoptosis induction in mammalian cells.

Our study reports the activation of caspase-3, -8, -9 and -12 upon EAV Bucyrus strain infection of the RK13 cell line. Whereas caspase-3 and -9 were detected in the three cell lines reported in the literature, divergent results have been found for caspase-8. Caspase-8 has been found activated in Vero (St-Louis and Archambault, 2007) and RK13 cells (present study), but not in BHK21 cells (Cholleti et al., 2013).

There are differences in the ability to induce apoptosis of different virus strains of other arteriviruses (Mu et al., 2015). Only the attenuated and non-pathogenic strain of rabies virus triggers caspase-dependent apoptosis in human cells (Préhaud et al., 2003).

Considering the viral strains used in published EAV apoptosis-studies, we could not conclude any correlation between caspase-8 activation and the pathogenic (SP3A and Bucyrus) or non-pathogenic (T1329 and Arvac) strains. As we detected caspase-8 activation in RK13 using the pathogenic Bucyrus EAV strain but this activation was absent using the pathogenic EAV SP3A strain in BHK21 cells, we could suggest that the activation of the extrinsic pathway is more related to the cell line rather than with the pathogenicity of the EAV strain. Thus, further studies of the effect of viral pathogenicity should be conducted in subsequent studies in the same cell line with different strains.

This is the first report of caspase-12 activation by EAV. Different studies have demonstrated the association of caspase-7 and caspase-12 with the ER compartment, and the translocation of cytosolic caspase-7 to the ER compartment to activate caspase-12 (Nakagawa and Yuan, 2000; Rao et al., 2001).

The results of the present work suggest that different apoptotic pathways and crosstalk between them may be involved in the induction of apoptosis in specific cell lines. In conclusion, EAV in RK13 cells was shown to induce extrinsic (caspase-8), intrinsic (caspase-9) and ER stress-induced apoptosis (caspase-12) pathways.

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