Regulation of ROS in transmissible gastroenteritis virus-activated apoptotic signaling

Li Ding [a,b,1], Xiaomin Zhao [a,1], Yong Huang [a], Qian Du [a], Feng Dong [a], Hongling Zhang [a], Xiangjun Song [a], Wenlong Zhang [a], Dewen Tong [a,*]

[a] College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, PR China
[b] College of Life Sciences, Hainan Normal University, Haikou, Hainan 571158, PR China

ABSTRACT

Transmissible gastroenteritis virus (TGEV), an enteropathogenic coronavirus, causes severe lethal watery diarrhea and dehydration in piglets. Previous studies indicate that TGEV infection induces cell apoptosis in host cells. In this study, we investigated the roles and regulation of reactive oxygen species (ROS) in TGEV-activated apoptotic signaling. The results showed that TGEV infection induced ROS accumulation, whereas UV-irradiated TGEV did not promote ROS accumulation. In addition, TGEV infection lowered mitochondrial transmembrane potential in PK-15 cell line, which could be inhibited by ROS scavengers, pyrrolidinedithiocarbamic (PDTC) and N-acetyl-L-cysteine (NAC). Furthermore, the two scavengers significantly inhibited the activation of p38 MAPK and p53 and further blocked apoptosis occurrence through suppressing the TGEV-induced Bcl-2 reduction, Bax redistribution, cytochrome c release and caspase-3 activation. These results suggest that oxidative stress pathway might be a key element in TGEV-induced apoptosis and TGEV pathogenesis.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Transmissible gastroenteritis virus (TGEV), a member of the family coronaviridae in the genus alphacoronavirus [1,2], could cause severe lethal watery diarrhea and dehydration in piglets and lead to serious economic loss for the pork industry. Previous studies indicated that TGEV infection could activate proapoptotic signaling to trigger cell apoptosis, and further result in CPE and cell death in permissive cells [3–7]. However, the upstream apoptotic signaling activated by TGEV infection is still not completely elucidated.

Oxidative stress imposed either directly by the virus or by the host-immune response, is a potentially important pathogenic mechanism in many virus infectious diseases [8–11]. Reactive oxygen species (ROS) are involved in various cellular processes such as proliferation, apoptosis and inflammation [12]. The generation of ROS can be induced under various conditions of stress, including ionizing radiation, chemotherapeutic drugs and viral infections [13]. ROS not only kill target cells but may also overwhelm cellular antioxidant defenses of neighboring cells, leading to damage of DNA [12]. Meanwhile, ROS have also been implicated in some important signaling pathways, such as MAPK and p53 pathways [14]. In our previous works we have demonstrated that TGEV

activates p38 MAPK and p53 signaling pathways, causes transcriptional induction of Bax and cytochrome c release from mitochondria to cytosol upon TGEV infection [6,7]. The study presented here demonstrated that TGEV infection could induce ROS accumulation to regulate the apoptotic signalings, which might play primary role in the pathogenesis of TGEV.

2. Materials and methods

2.1. Antibodies, cells and virus

Monoclonal antibodies against cytochrome c, Bax, Bcl-2, β-actin, Cox4, p53, p38, p-p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, Inc., CA, US). Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Pierce (Pierce, Rockford, IL, US). PK-15 cells (ATCC, CCL-33) were grown in Dulbecco Minimal Essential Medium (D-MEM) (Gibco BRL, MD, US) supplemented with 10% heat-inactivated new born bovine serum (Gibco BRL, MD, US), 100 IU of penicillin and 100 μg of streptomycin per ml, at 37 °C in humidified 5% CO_2. The TGEV Shaanxi strain was used as previously described [6,7].

2.2. Observation of ROS production under fluorescence microscopy

Reactive oxygen species (ROS) were detected using the cell-permeable fluorescent-probe 2′-7′-dichlorofluorescein diacetate

* Corresponding author. Fax: +86 29 87091032.
  E-mail address: dwtong@nwsuaf.edu.cn (D. Tong).
[1] These authors contributed equally to the work.

(DCFH–DA) (Sigma–Aldrich, US). Briefly, cells were grown in 24-well tissue culture plates, and the cells were incubated with 10 μM DCFH-DA at 37 °C for 1 h in the dark, and then infected with TGEV at 10 MOI for different time. The cells were observed under a fluorescence microscope (AMG EVOS Inc., Israel).

2.3. ROS measurement

Mock-infected and TGEV-infected cells were harvested at indicated times. After washed twice with PBS, the cells were incubated with 10 μM DCFH-DA at 37 °C for 1 h in the dark for final analysis by flow cytometry (Beckman Coulter EPICS ALTRA, Orlando, US).

2.4. Mitochondrial transmembrane potential (Δψm) assessment

The Δψm was analyzed using a JC-1 Mitochondrial Potential Detection Kit (Biotium Inc., Hayward, CA, US). Briefly, cells were harvested at indicated times and stained by JC-1 in PBS for 15 min at room temperature in the dark, followed by flow cyto-metric analysis.

2.5. Western blot analysis

Cell extracts were prepared as described previously (Ding et al., 2012). Protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, US). Equivalent amounts of proteins were loaded and electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Subsequently, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp, Atlanta, GA, US). The membranes were blocked with 5% nonfat dry milk at room temperature for 1 h, and then incubated with indicated primary antibodies overnight at 4 °C, followed by HRP-conjugated secondary antibodies at room temperature for 1 h. The signal was detected using ECL reagent (Pierce, Rockford, IL, US).

2.6. Caspase-3 activity assay

Caspase-3 activity was measured by colorimetric assay kit (BioVision, Inc., CA, US) following the manufacture's instructions. Briefly, cell lysates were prepared and protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL,
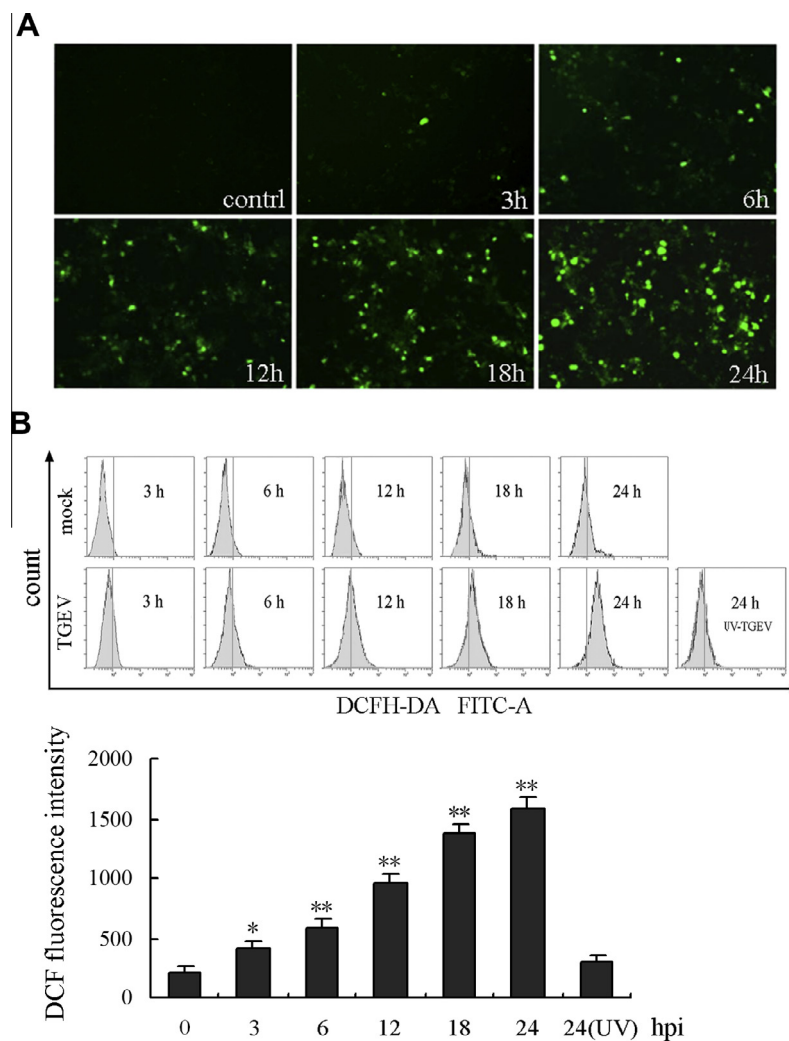

Fig. 1. TGEV infection induced ROS production. (A) Observation of ROS production under Fluorescence microscopy. PK-15 cells were grown in 24-well cell culture plates, and the cells were incubated with 10 μM DCFH-DA at 37 °C for 1 h in the dark, and then infected with TGEV at a MOI of 10 for different time. The cells were observed under a fluorescence microscope. (B) Flow cytometric analysis of ROS in TGEV-infected PK-15 cells. Cells infected with TGEV at 10 MOI for different times or infected with UV-TGEV at 24 h were harvested, and then stained by DCFH-DA dye. The fluorescence intensity of stained cells was determined by flow cytometry (upper panel). The levels of ROS for each infectious time were calculated by the fluorescence intensity in TGEV-infected cells subtracting the fluorescence intensity in mock-infected cells (lower panel). Values are mean ± SD. *P < 0.05, **P < 0.01 versus 0 h.

US). Then 200 μg of protein in each sample was incubated with caspase-3 substrate at 37 °C for 4 h. Samples were read at 405 nm in microplate spectrophotometer (BioTek Instruments, Inc., Winooski, US).

2.7. Apoptotic rate measurement

Annexin V-FITC Apoptosis Kit (BioVision, Inc., CA, US) was used for apoptosis detection according to the manufacturer's protocol. Briefly, cells were washed twice with PBS and resuspended in 500 μl binding buffer, followed by adding 5 μl of Annexin V-FITC and 5 μl of PI. After incubation in the dark for 30 min at room temperature, a total of 10 000 cells were acquired and percentage of positive cells were analyzed by flow cytometry (Beckman Coulter, Inc. Fullerton, CA. US).

3. Results

3.1. TGEV infection induced ROS accumulation

To explore the upstream mechanism of TGEV-induced cell damage, we measured ROS levels in TGEV-infected PK-15 cells using DCFH-DA, a non-fluorescent compound, which is converted into highly fluorescent dichlorofluorescein (DCF) by cellular peroxides [15]. Results showed that the fluorescence in PK-15 cells was observed at 3 h post infection (p.i.), and the fluorescence intensity enhanced with infectious time increase (Fig. 1A). To further confirm the ROS production, intracellular ROS production was analyzed by flow cytometry. As shown in Fig. 1B, consistent with the observation under fluorescence microscopy, TGEV infection induced an increase in DCF fluorescence (Fig. 1B, upper panel). At 3, 6 and 12 h post-infection, ROS production in TGEV-infected cells increased markedly compared with that of mock-infected cells, and maintained a higher level in next infectious time (Fig. 1B, lower panal). However, UV-inactivated TGEV lost their ability to increase ROS production (Fig. 1B). These results suggest that TGEV induce ROS accumulation in PK-15 cells.

3.2. TGEV infection reduced mitochondrial membrane potential

Since ROS could stimulate the intrinsic mitochondrial apoptotic pathway and mitochondrial DNA damage to promote outer membrane permeabilization [12], we used JC-1 as fluorescence probe to evaluate changes in mitochondrial membrane potential ($\Delta\psi m$) by flow cytometry. JC-1 forms monomers and emits green fluorescence at low $\Delta\psi m$, while it forms aggregates and emits red fluorescence at high $\Delta\psi m$. Fig. 2A showed that the $\Delta\psi m$-depolarized cells increased about 26.2% and 61.3% at 12 and 24 h in TGEV infected PK-15 cells, respectively, compared with mock infection. Two different antioxidants of ROS, pyrrolidinedithiocarbamic (PDTC) (Merck KGaA, Darmstadt, Germany) and N-acetyl-L-cysteine (NAC) (Sigma–Aldrich, US), which did not show cytotoxicity at concentrations used (data not shown), significantly prevented the production of ROS and reduction of mitochondrial membrane potential in TGEV-infected cells (Fig. 2B and C). These results indicated that TGEV infection might reduce mitochondrial membrane potential through the production of ROS.

3.3. Effects of ROS on TGEV-induced apoptosis

Mitochondria are crucial to regulate apoptosis upon virus infection. To further explore the roles of ROS accumulation in TGEV-induced apoptosis, we investigated the effects of PDTC and NAC, on the expression of Bcl-2, Bax and cytochrome c redistribution, caspase-3 activity and apoptosis induction. As predicted, the

expression of Bcl-2 increased (Fig. 3A), Bax and cytochrome c (Cyt c) redistribution was attenuated evidently in mitochondria and cytosol respectively in TGEV-infected PK-15 cells with PDTC- and NAC treatment (Fig. 3B). In addition, caspase-3 activity was remarkably decreased in the TGEV-infected cells with PDTC and NAC compared to that without the two inhibitors (Fig. 3C). Consequently, PDTC and NAC treatment significantly suppressed TGEV-induced apoptosis in PK-15 cells (Fig. 3D). These results suggest that ROS play a pivotal role in the process of apoptosis induced by TGEV.

3.4. ROS might promote the activation of p38 MAPK and p53 in TGEV-infected PK-15 cells

In our previous study, we found that TGEV could regulate apoptosis by inducing p38 MAPK and p53 pathways [7]. ROS may play
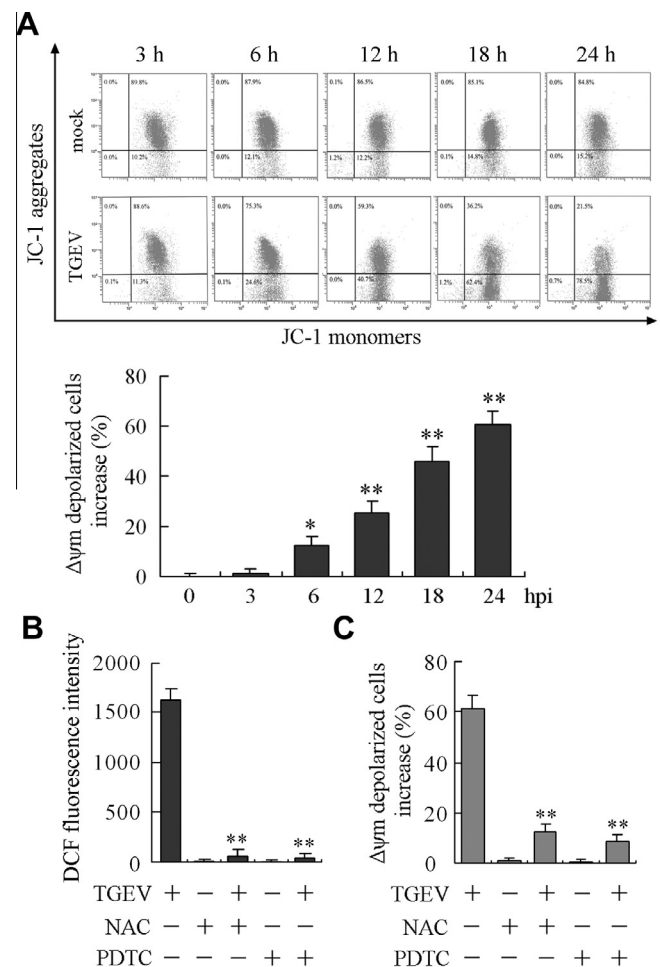

Fig. 2. ROS accumulation reduced mitochondrial membrane potential in TGEV infected PK-15 cells. (A) TGEV infection induced collapse of $\Delta\psi m$. Cells were infected with 10 MOI of TGEV for different times, and stained with JC-1 for 15 min at room temperature, followed by FCM analysis. The increase of $\Delta\psi m$-depolarized cells was that $\Delta\psi m$-depolarized cells in TGEV-infected cells subtracted $\Delta\psi m$-depolarized cells in mock-infected cells. *$P < 0.05$, **$P < 0.01$ versus 0 h. (B) Effects of ROS scavengers (NAC and PDTC) on ROS production in TGEV-infected PK-15 cells. The cells were infected with TGEV for 24 h, in the presence or absence of NAC (10 mM) or PDTC (100 μM), and the ROS production was analyzed by flow cytometry. The levels of ROS were that the fluorescence intensity in different treated-cells subtracted the fluorescence intensity in control cells. **$P < 0.01$ versus TGEV infection without NAC and PDTC. (C) Effects of ROS scavengers (NAC and PDTC) on $\Delta\psi m$ in TGEV-infected PK-15 cells. The cells were infected with TGEV for 24 h, in the presence or absence of NAC (10 mM) or PDTC (100 μM), and the $\Delta\psi m$ were analyzed by flow cytometry. The increase of $\Delta\psi m$-depolarized cells was that $\Delta\psi m$-depolarized cells in different treatment subtracted $\Delta\psi m$-depolarized cells in the control. **$P < 0.01$ versus TGEV infection without NAC and PDTC.

an important role in the activation of these signaling pathways, thus we detected whether ROS were involved in TGEV-induced p38 MAPK and p53 activation. PK-15 cells were pre-treated with the scavenger NAC and PDTC for 1 h, and then co-treated with TGEV, the levels of p38 MAPK and p53 were evaluated by Western blot. As shown in Fig. 4A, two ROS scavengers treatment significantly reduced p53 protein levels and the phosphorylation of p38 MAPK when TGEV infected PK-15 cells for 24 h, suggesting that ROS might be involved in p38 MAPK and p53 activation upon TGEV infection.

In order to investigate whether the activation of p38 MAPK and p53 pathways promote ROS production, we tested ROS levels in TGEV-infected PK-15 cells with specific p53 inhibitor pifithrin-α (PFT-α) (Sigma–Aldrich, US) or p38 MAPK inhibitor SB203580 (Merck KGaA, Darmstadt, Germany). Fig. 4B showed that the presence of PFT-α and SB203580 resulted in decrease of ROS production in a certain extent, especially PFT-α had more significant effect on TGEV induction of ROS production, suggesting that the activation of p53 might be involved in ROS production in TGEV-infected PK-15 cells. Taken together, these results suggest that TGEV-induced ROS production might promote the activation of p38 MAPK and p53, and that p53, in turn, might partly regulate ROS production.

4. Discussion

Oxidative stress has been known as a mediator of apoptosis induced by a variety of triggers, including viral infection [8,9]. Previous study indicated that an abnormal cellular oxidation event occurred during TGEV infection, which might account for cell injury and apoptosis induction [4]. To probe the relation between TGEV and oxidative stress, we investigated the direct effect of the viral infection on the oxidative profile of TGEV host cells during the stages of infection. The results showed that TGEV infection induced ROS accumulation, which played a key role in TGEV-induced apoptosis.

Recently, mitochondria are regarded as sensors of oxidation damage and may play a major role in apoptosis [14,16]. Apoptosis can be preceded by loss of selective ion permeability, leading to the formation of mitochondrial permeability transition pores and the release of apoptosis initiating factors, triggering the activity of
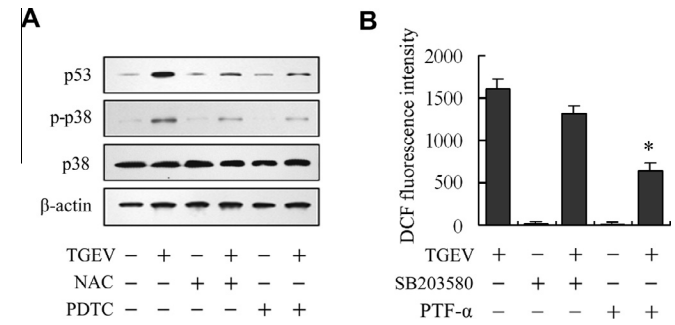
Fig. 4. TGEV induction of ROS production is involved in p38 MAPK and p53 activation in PK-15 cells. (A) Effects of ROS scavengers on the activation of p38 MAPK and p53. Cells were pretreated with ROS specific scavengers NAC and PDTC for 1 h, and then co-treated with TGEV for 24 h. Cell lysates were analyzed by Western blot. (B) Effects of p38 MAPK and p53 inhibitors on ROS generation. Cells were pretreated with p38 MAPK specific inhibitor SB203580 (20 μM) and p53 specific inhibitor PTF-α (20 μM) for 1 h, and then co-treated with TGEV for 24 h. Cells were harvested, and stained by DCFH-DA dye, then analyzed by flow cytometry. The levels of ROS were that the fluorescence intensity in different treated-cells subtracted the fluorescence intensity in control cells. Values are mean ± SD. *$P < 0.05$ versus TGEV infection without inhibitors.

caspases [14]. Time course analysis of TGEV infected PK-15 cells showed that with ROS accumulation, mitochondrial membrane potential reduced significantly, suggesting that TGEV-induced ROS accumulation lead to outer membrane permeabilization (MOMP). Mitochondrial oxidative damage can also increase the tendency of mitochondria to release cellular toxic proteins such as cytochrome c (cyt c) to the cytosol and thereby activate the cell's apoptotic machinery [14,17]. In the present study, Two ROS scavengers (PDTC and NAC) significantly inhibited TGEV infection-induced Bax redistribution, cytochrome c release, caspase-3 activation, and apoptosis occurrence, suggesting that ROS play a pivotal role in the process of TGEV induction of cell apoptosis.

ROS have also been reported that could be involved in some important signaling pathways, such as MAPK and p53 pathways [14,18,19]. In our present works we have demonstrated that about 60% of ROS were produced after p53 signaling was activated in TGEV infected PK-15 cells. Block of p53 activation could significantly attenuate the increase of this part of ROS. These results
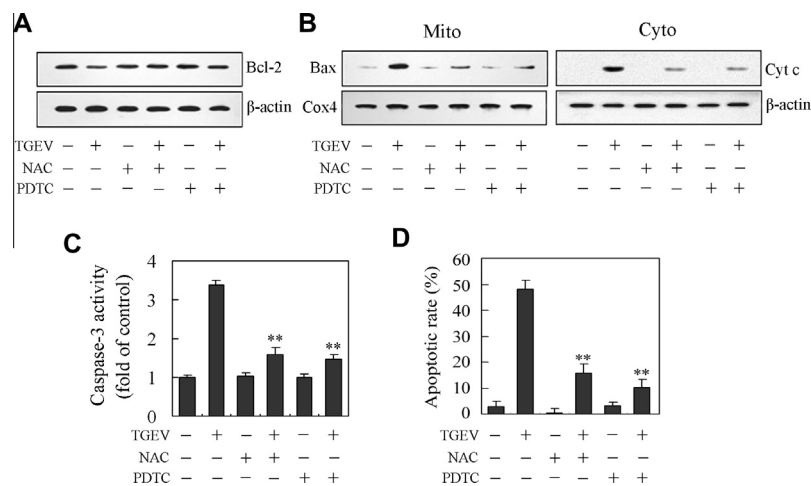
Fig. 3. Effects of NAC and PDTC on apoptosis in TGEV-infected PK-15 cells. (A and B) Effect of NAC and PDTC on expression of Bcl-2, translocation of Bax and release of cytochrome c. The cells were infected with TGEV for 24 h, in the presence or absence of NAC or PDTC. Cell lysates were analyzed by Western blot. Cox4 and β-actin were used as internal controls for the mitochondrial fractions and the cytosolic fractions, respectively. (C) Effect of NAC and PDTC on the activity of caspase-3. Cells were pretreated with NAC and PDTC for 1 h, and then co-treated with TGEV for 24 h. Caspase-3 activity was measured by colorimetric assay kit. Values are mean ± SD. **$P < 0.01$ versus TGEV infection without antioxidants. (D) Effect of NAC and PDTC on apoptotic rate in TGEV-infected PK-15 cells. The cells were infected with TGEV for 24 h in the presence or absence of NAC and PDTC, and the apoptotic ratio was analyzed by flow cytometry. Values are mean ± SD. **$P < 0.01$ versus TGEV infection without NAC and PDTC.

suggest that ROS accumulation in TGEV-infected cells is predominantly attribute to p53 activation. In addition, inhibition of ROS by NAC and PDTC could partially reduce the activation of p38 MAPK and p53, suggesting that ROS might be an upstream signal molecule involved in the activation of p38 MAPK and p53 signaling pathways in TGEV-infected PK-15 cells. Our results provide a link between the known mediators of apoptosis, p53 and ROS, and indicate the order in the pathway by which these mediators exert their effects ROS downstream to p53. Consistent with this model, ROS production in response to p53 occurred ahead of apoptotic death, indicating that p53-generated ROS may constitute a signal for apoptosis rather than being a consequence of the cellular changes associated with apoptosis.

In conclusion, the current investigation demonstrated that in vitro TGEV infection leads to ROS accumulation in PK-15 cells. The oxidative stress may contribute to the alteration of the intracellular events leading to apoptosis.

5. Contributors and authorship

Li Ding and Xiaomin Zhao designed the experiments, interpreted the data and wrote the article. Li Ding performed the experiments with assistance and advice from Xiaomin Zhao, Qian Du, Feng Dong, Hongling Zhang, Xiangjun Song, Wenlong Zhang. Yong Huang and Dewen Tong revised the manuscript. All authors have read the manuscript and approved to submit it to your journal.

Acknowledgments

References

[1] E. Carstens, Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses, Archives of Virology 155 (2009) (2010) 133–146.

[2] A. Lorusso, N. Decaro, P. Schellen, P.J. Rottier, C. Buonavoglia, B.-J. Haijema, R.J. de Groot, Gain, preservation, and loss of a group 1a coronavirus accessory glycoprotein, Journal of Virology 82 (2008) 10312–10317.

[3] J.-F. Eléouët, E.A. Slee, F. Saurini, N. Castagné, D. Poncet, C. Garrido, E. Solary, S.J. Martin, The viral nucleocapsid protein of transmissible gastroenteritis coronavirus (TGEV) is cleaved by caspase-6 and -7 during TGEV-induced apoptosis, Journal of Virology 74 (2000) 3975–3983.

[4] J.-F. Eleouet, S. Chilmonczyk, L. Besnardeau, H. Laude, Transmissible gastroenteritis coronavirus induces programmed cell death in infected cells through a caspase-dependent pathway, Journal of Virology 72 (1998) 4918–4924.

[5] T. Sirinarumitr, J. Kluge, P. Paul, Transmissible gastroenteritis virus induced apoptosis in swine testes cell cultures, Archives of Virology 143 (1998) 2471–2485.

[6] L. Ding, X. Xu, Y. Huang, Z. Li, K. Zhang, G. Chen, G. Yu, Z. Wang, W. Li, D. Tong, Transmissible gastroenteritis virus infection induces apoptosis through FasL- and mitochondria-mediated pathways, Veterinary Microbiology 158 (2012) 12–22.

[7] Y. Huang, L. Ding, Z. Li, M. Dai, X. Zhao, W. Li, Q. Du, X. Xu, D. Tong, Transmissible gastroenteritis virus infection induces cell apoptosis via activation of p53 signalling, Journal of General Virology 94 (2013) 1807–1817.

[8] M. Korenaga, T. Wang, Y. Li, L.A. Showalter, T. Chan, J. Sun, S.A. Weinman, Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production, Journal of Biological Chemistry 280 (2005) 37481–37488.

[9] B. Gruhne, R. Sompallae, D. Marescotti, S.A. Kamranvar, S. Gastaldello, M.G. Masucci, The Epstein–Barr virus nuclear antigen-1 promotes genomic instability via induction of reactive oxygen species, Proceedings of the National Academy of Sciences of the United States of America 106 (2009) 2313–2318.

[10] M.A. Torres, J.D. Jones, J.L. Dangl, Reactive oxygen species signaling in response to pathogens, Plant Physiology 141 (2006) 373–378.

[11] W.H. Tung, H.W. Tsai, I.T. Lee, H.L. Hsieh, W.J. Chen, Y.L. Chen, C.M. Yang, Japanese encephalitis virus induces matrix metalloproteinase-9 in rat brain astrocytes via NF-κB signalling dependent on MAPKs and reactive oxygen species, British Journal of Pharmacology 161 (2010) 1566–1583.

[12] M.L. Circu, T.Y. Aw, Reactive oxygen species, cellular redox systems, and apoptosis, Free Radical Biology and Medicine 48 (2010) 749–762.

[13] T. Finkel, Signal transduction by reactive oxygen species, The Journal of Cell Biology 194 (2011) 7–15.

[14] R.B. Hamanaka, N.S. Chandel, Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes, Trends in Biochemical Sciences 35 (2010) 505–513.

[15] E. Eruslanov, S. Kusmartsev, Identification of ROS using oxidized DCFDA and flow-cytometry, Springer, Advanced Protocols in Oxidative Stress II, 2010. pp. 57–72.

[16] H.-U. Simon, A. Haj-Yehia, F. Levi-Schaffer, Role of reactive oxygen species (ROS) in apoptosis induction, Apoptosis 5 (2000) 415–418.

[17] T.-C. Yang, C.-C. Lai, S.-L. Shiu, P.-H. Chuang, B.-C. Tzou, Y.-Y. Lin, F.-J. Tsai, C.-W. Lin, Japanese encephalitis virus down-regulates thioredoxin and induces ROS-mediated ASK1-ERK/p38 MAPK activation in human promonocyte cells, Microbes and Infection 12 (2010) 643–651.

[18] J. Huang, L. Wu, S.-I. Tashiro, S. Onodera, T. Ikejima, Reactive oxygen species mediate oridonin-induced HepG2 apoptosis through p53, and mitochondrial signaling pathways, Journal of Pharmacological Sciences 107 (2008) 370–379.

[19] P. Bragado, A. Armesilla, A. Silva, A. Porras, Apoptosis by cisplatin requires p53 mediated p38α MAPK activation through ROS generation, Apoptosis 12 (2007) 1733–1742.