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Rapid Communication

Importance of Akt signaling pathway for apoptosis in SARS-CoV-infected Vero E6 cells

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Abstract

Severe acute respiratory syndrome (SARS) is an acute respiratory tract infectious disease that is associated with a new coronavirus (SARS-CoV). Our recent study indicated that SARS-CoV infection induces activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway and the p38 MAPK inhibitor partially inhibited its cytopathic effect in Vero E6 cells. The results of the present study indicated that before cell death, Akt, which is an inhibitor of apoptosis, was also activated in response to viral replication. Phosphorylation of a serine residue on Akt was detected at least 8 h postinfection (hpi), which declined after 18 hpi. Thus, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is activated in virus-infected Vero E6 cells. However, a threonine residue was not phosphorylated. A downstream target of Akt, glycogen synthase kinase 3β (GSK-3β), was slightly phosphorylated, indicating that the level of activation of Akt was very low. PKCζ, which is downstream of the PI3K pathway, was also phosphorylated in virus-infected cells. These results suggested that weak activation of Akt cannot prevent apoptosis induced by SARS-CoV infection in Vero E6 cells.
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Keywords: SARS-CoV; Akt; PI3K; PKC

Severe acute respiratory syndrome (SARS) is a newly described infectious disease caused by a coronavirus (Rota et al., 2003). Although the mechanism of SARS pathogenesis in vivo may involve both the effect of viral replication in target cells and immune responses, there is a lack of molecular pathological data including signaling pathways of SARS-coronavirus (CoV) infection. Recently, we reported that infection with SARS-CoV caused apoptosis in Vero E6 cells and that p38 mitogen-activated protein kinase (MAPK) was activated during infection (Mizutani et al., 2004). Thus, it is important to investigate the apoptotic events that occur in SARS-CoV-infected cells to understand the pathogenesis of SARS-CoV. Generally, both pro-apoptotic and pro-survival signaling pathways are activated after apoptotic signaling, and there are many pro- and anti-apoptotic proteins involved in these pathways in cells. The present study was performed to clarify the activated signaling pathways related to apoptotic events in SARS-CoV-infected Vero E6 cells.

In SARS-CoV-infected Vero E6 cells, cytopathic effects were observed within 24 h postinfection (hpi). We have recently shown that p38 MAPK and its downstream targets are phosphorylated in SARS-CoV-infected Vero E6 cells (Mizutani et al., 2004). These findings suggest that other signaling pathways are also activated in SARS-CoV-infected cells. To investigate such cellular responses in SARS-CoV-infected Vero E6 cells before apoptosis, proteins of SARS-CoV-infected and mock-infected cells were analyzed by Western blotting analysis at 18 hpi using 125 antibodies to human cellular proteins. We found that antibodies to proteins related to several signaling pathways responded specifically in SARS-CoV infection (\ below and unpublished data).

One such signaling pathway, protein kinase B (PKB), known as Akt, has been studied intensively (Toker, 2000;

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Brazil and Hemmings, 2001; Scheid and Woodgett, 2003). Akt is phosphorylated at both serine 473 and threonine 308 residues through a phosphatidylinositol 3-kinase (PI3K)-dependent mechanism on stimulation by growth factors, insulin, and hormones (Welch et al., 1998). An important function of activated Akt in cells is inhibition of apoptosis. Downstream targets of Akt induce cell survival via phosphorylation of the forkhead transcription factor (FKHR) family, glycogen synthase kinase 3β (GSK-3β), caspase-9, and Bad (Cardone et al., 1998; Cross et al., 1995; Datta et al., 1997). To examine the phosphorylation of Akt in virus-infected cells, Western blotting analysis was performed using anti-phospho Akt antibodies. As shown in Fig. 1A, serine 473 of Akt was phosphorylated at 8 hpi by SARS-CoV. The phosphorylated serine 473 of Akt began to accumulate at least 8 hpi and maximal phosphorylation was observed at 18 hpi. Western blotting analysis with a phosphorylation-state-independent anti-Akt antibody revealed the presence of Akt in Vero E6 cells, whereas a threonine 308 phosphorylation-state-dependent antibody failed to demonstrate the presence of threonine 308 phosphorylated Akt in virus-infected cells (Fig. 1A). In addition, serine phosphorylation was dependent on SARS-CoV infection into Vero E6 cells, because the UV-inactivated virus failed to induce serine phosphorylation. Brief stimulation by epidermal growth factor (EGF) also showed only serine 473 phosphorylation of Akt (Fig. 1B), suggesting that threonine 308 of Akt is difficult to phosphorylate in Vero E6 cells. Therefore, total activity of Akt may be low in Vero E6 cells. However, it is possible that the anti-phospho Akt (threonine 308) antibody used in the present study is unable to recognize threonine phosphorylation of Akt in Vero E6 cells as the datasheet included no description of cross-reactivity with monkey. To investigate whether SARS-CoV-induced Akt serine phosphorylation represented a biologically active kinase, we examined the in vitro kinase activity of phosphorylated Akt in SARS-CoV-infected cells. The SARS-CoV-infected Vero E6 cells were lysed at 18 hpi and serine 473-phosphorylated Akt in the cell lysate was precipitated by anti-serine 473-phosphorylated Akt antibody. GSK-3β protein was added to the immunoprecipitated phosphorylated Akt with ATP, and Western blotting was performed using anti-phosphorylated GSK-3α/β (Ser21/9) antibody. As shown in Fig. 1C, immunoprecipitated Akt was almost at the same amount between SARS-CoV-infected cells and mock-infected cells at 18 hpi. The amount of serine 473-phosphorylated Akt in SARS-CoV-infected cells was higher than that in mock-infected controls. The level of phosphorylation of GSK-3α/β in virus-infected cells was slightly higher than that in mock-infected controls. These results strongly suggested that Akt in Vero E6 cells was phosphorylated only at serine residues by SARS-CoV infection, but the level of activity of Akt was low.

To investigate phosphorylation of up- and downstream targets of Akt in virus-infected cells, Western blotting analysis was performed. Of the Akt targets mentioned above, the level of phosphorylation of GSK-3β (Ser9) was slightly increased, while phosphorylated Bad and FKHR were not detected (Fig. 1C). GSK-3 β is a pro-apoptotic signaling molecule and is inactivated by phosphorylation of the N-terminal serine residue Ser9 (Pap and Cooper, 1998).

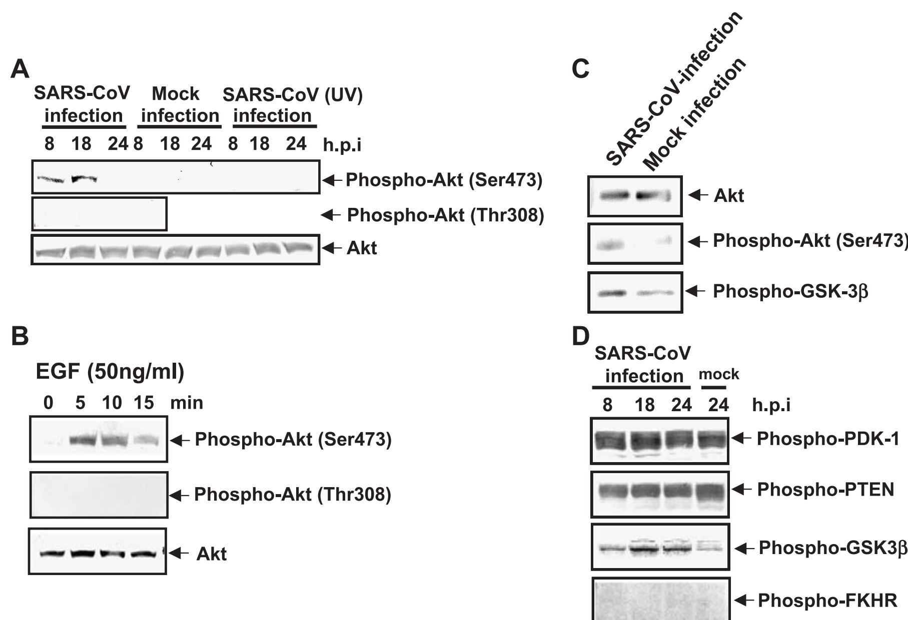

Fig. 1. Phosphorylation of Akt in SARS-CoV-infected Vero E6 cells. (A) Vero E6 cells were infected with SARS-CoV at moi of 5, and Western blotting was then performed using proteins obtained at 8, 18, and 24 hpi. FCS-free medium and UV-inactivated SARS-CoV were used as controls. Phosphorylated Akt was detected by Western blotting analysis. (B) EGF was added to Vero E6 cells for 5, 10, and 15 min. (C) In vitro Akt kinase assay was performed using proteins from SARS-CoV-infected Vero E6 cells and mock-infected cells at 18 hpi. (D) Phosphorylation of up- and downstream targets of Akt was detected by Western blotting analysis.

Therefore, these results suggested that the phosphorylation of Akt in Vero E6 cells results in a low level of anti-apoptotic response to SARS-CoV infection. Recent studies explained the activation cycle of Akt as outlined below (Toker, 2000; Brazil and Hemmings, 2001; Scheid and Woodgett, 2003). Activation of PI3K results in local accumulation of phosphatidylinositol (PtdIns)-3,4,5-triphosphate (P3) at the plasma membrane, and PTEN phosphatase inhibits PI3K signaling. Cytosolic Akt is inactive and the activity of cytosolic PDK-1 is low. PtdIns-3,4,5-P3 recruits PDK-1 and Akt to the plasma membrane, and then Akt is autophosphorylated at serine 473. After PDK-1 activation on the plasma membrane, PDK-1 phosphorylates Akt on threonine 308, and then Akt shows a high level of activity. Thus, activation of upstream kinases of Akt is important for the activation of Akt. Fig. 1C shows that the amounts of both phosphorylated PTEN and PDK-1 were not significantly altered in SARS-CoV-infected Vero E6 cells. Therefore, these results may explain why no increase in threonine 308 phosphorylation of Akt was observed on virus infection.

The PI3 kinase inhibitor LY294002 is widely used to study Akt phosphorylation in stimulations in vitro. We examined whether LY294002 inhibits serine phosphorylation of Akt induced by SARS-CoV infection. Vero E6 cells were treated with LY294002 (10 and 20 μM) for 1.5 h, and then infected with SARS-CoV. Western blotting analysis was performed on the cell lysate at 18 hpi. As shown in Fig. 2A, the serine residue of Akt was not phosphorylated in cells treated with either concentration of LY294002. This result suggested that serine phosphorylation of Akt was sensitive to the PI3K inhibitor and that PI3K was activated in virus-infected cells. In addition, DNA fragmentations in infected Vero E6 cells at 30 hpi were similar in the presence and absence of LY294002 (Fig. 2B), suggesting that weak activation of Akt cannot prevent apoptosis induced by SARS-CoV infection in Vero E6 cells.

As shown in Fig. 2A, the level of viral N protein expression seemed to be similar at 18 hpi among cells treated with various amounts of LY294002. Next, we investigated whether enhancement of virus-specific protein synthesis through Akt-serine 473 phosphorylation occurs in SARS-CoV-infected cells at early time points pi. Recently, Simmons et al. (2004) reported that acidification of endosomes was required for SARS-CoV S-mediated viral entry using S glycoprotein pseudoviruses, after viral adsorption to angiotensin-converting enzyme (ACE)2 (Li et al., 2003). They reported that SARS-CoV S protein is cleaved in the presence of trypsin and that the cleaved S induces cell fusion. Their results suggested that SARS-CoV enters cells via endocytosis. Before a virus infection experiment, to verify that LY294002 has no effect on acidification of intracellular compartments in Vero E6 cells, LY294002 was added to the cells, followed by staining with acridine orange to detect intracellular acidic compartments (Mizutani et al., 2003). As indicated in Fig. 2C, neither 10 nor 20 μM LY294002 had any effect on the acidification of intracellular compartments, suggesting that treatment with LY294002 did not inhibit endocytosis in Vero E6 cells. Next, Western blotting analysis was performed at 6, 9, and 12 hpi using anti-N antibody. As shown in Fig. 2D, the kinetics of SARS-CoV-N protein accumulation in infected Vero E6 cells were similar in the presence and absence of LY294002. These

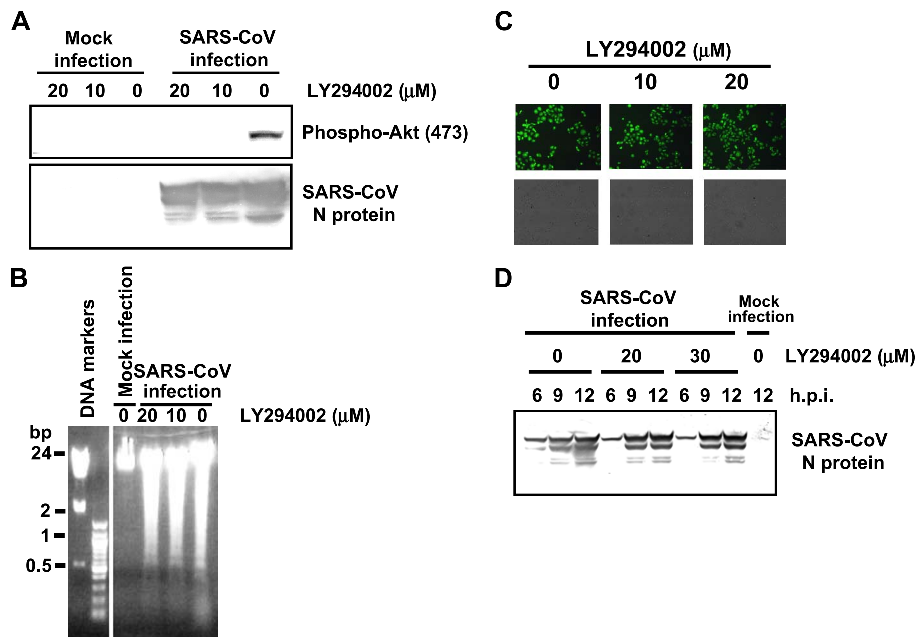

Fig. 2. Effects of LY294002 on SARS-CoV infection. (A) Vero E6 cells were pretreated with LY294002 and then infected with SARS-CoV at moi of 5. Western blotting analysis was performed at 18 hpi. (B) Cellular DNA was extracted from Vero E6 cells in the presence or absence of LY294002 at 30 hpi. (C) Vero E6 cells were incubated for 1.5 h with LY294002 and stained with acridine orange. (D) Western blotting analysis was performed at 6, 9, and 12 hpi for detection of SARS-CoV N protein.

results indicated that Akt serine phosphorylation induced by SARS-CoV infection had no effect on viral replication.

Protein kinase C (PKC) is also a major cellular mediator of biological functions. The PKC superfamily is divided into sub-superfamilies according to their activation profiles; conventional PKC (cPKC α, βI, βII, γ novel PKC (nPKC δ, ε, η, θ), atypical PKC (aPKCζ, ι/λ), PKCμ/PKD, and PKCυ (Toker, 2000). PKCζ was originally discovered as a unique PKC isotype (Ono et al., 1989). B cell survival by nerve growth factor (NGF) is mediated by PI3K-dependent activation of PKCζ (Kronfeld et al., 2002) and Akt could interact with PKCζ (Konishi et al., 1994). To determine if PKCζ is phosphorylated by SARS-CoV infection, Western blotting analysis was performed using an anti-phospho PKCζ (Thr410) antibody. As shown in Fig. 3, PKCζ was phosphorylated at 8 hpi, suggesting that PKCζ is activated as an anti-apoptotic response to virus infection. The antibody used in the present study can also detect phosphorylated PKCλ, and the function of PKCζ is still not clear. A novel PKC subfamily PKCθ was also very slightly phosphorylated by virus infection. In addition, PKCα/βII and δ (Ser643) were always phosphorylated in both virus-infected and mock-infected cells. These results suggested that the interaction of phosphorylation of PKCζ with Akt plays an important role in protection against SARS-CoV infection (Fig. 4).

In the present study, we showed that the PI3K/Akt pathway including PKCζ is activated by SARS-CoV infection, but that the level of activation of Akt in infected cells is very low. In virus infection, two conflicting cellular programs are triggered in cells: apoptosis to eliminate virus-infected cells and cell survival delaying cell death to alert naive cells by producing antiviral cytokines. This raises the question of how cell survival or death of SARS-CoV-infected cells is determined. The control mechanisms that balance cell survival against cell death are not well understood.

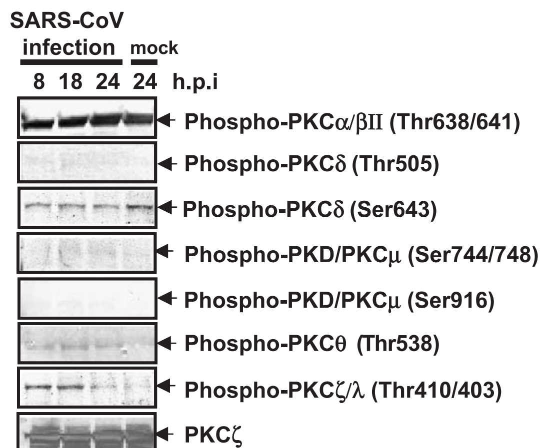

Fig. 3. Phosphorylation of PKCs in SARS-CoV-infected Vero E6 cells. Phosphorylated PKCs were detected by Western blotting analysis using proteins isolated from SARS-CoV-infected Vero E6 cells at 8, 18, and 24 hpi.

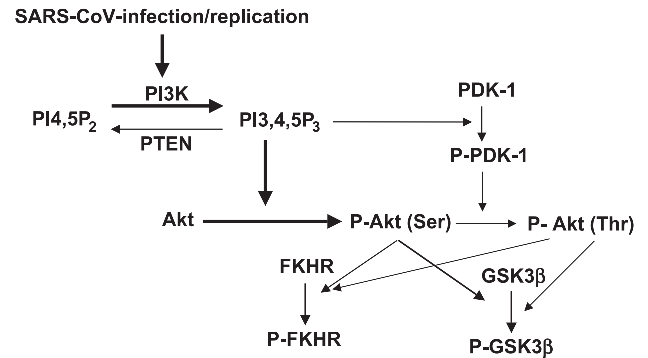

Fig. 4. Phosphorylation of Akt/PI3K signaling pathway in SARS-CoV-infected Vero E6 cells. Bold lines indicate activated pathways in viral-infected cells. Week or no activated pathways are indicated by thin lines.

Previously, we showed that activation of the p38 MAPK signaling pathway induced by SARS-CoV infection has a partially pro-apoptotic role in Vero E6 cells (Mizutani et al., 2004). The role of p38 MAPK signaling in cellular responses is diverse depending on the cell type and stimulus. For example, p38 MAPK signaling has been shown to promote cell death as well as to enhance cell growth and survival (Juretic et al., 2001; Liu et al., 2001; Yosimichi et al., 2001). In virus-infected Vero E6 cells, CREB and HSP-27, which have anti-apoptotic roles, were shown to be phosphorylated (Mizutani et al., 2004). Therefore, pro-apoptotic molecules downstream of targets of p38 MAPK may exist in Vero E6 cells. On the other hand, Akt, which is a key regulator of cell survival events, targets several different cytoplasmic proteins, including pro-apoptotic molecules, such as GSK3β, caspase-9, Bad, and FKHR. Phosphorylation of these proteins by Akt generally results in their inactivation and inability to activate pro-apoptotic pathways. In the present study, phosphorylation of GSK3β serine 9 was found in virus-infected cells. However, our results suggested that the level of phosphorylation of GSK3β is not sufficient to prevent apoptosis.

In conclusion, incomplete activation of Akt induces apoptosis in SARS-CoV-infected Vero E6 cells. However, we cannot exclude the possibility of the involvement of other signaling pathways that strongly induce apoptosis. These findings may facilitate the development of a new way to block apoptosis upstream and help in the development of anti-SARS-CoV agents.

Materials and methods

Cells and virus

Vero E6 cells were routinely subcultured in 75-cm^3 flasks in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 0.2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) fetal bovine serum (FBS), and maintained at

37 °C in an atmosphere of 5% CO_2. For use in the experiments, the cells were split once they reached 90% confluence and seeded onto 6- or 24-well tissue culture plate inserts. The culture medium was changed to 2% FBS containing DMEM before virus infection. In the present study, we used SARS-CoV (Drosten et al., 2003), which was isolated as Frankfurt 1 (Thiel et al., 2003) and kindly provided by Dr. J. Ziebuhr. Infection was usually performed with a multiplicity of infection (moi) of 5.

Western blotting

After virus infection, whole-cell extracts were electrophoresed in 12.5% and 10–20% gradient polyacrylamide gels, and transferred onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA). We applied two sets of samples to polyacrylamide gels, and the blots were divided into two sheets. The following antibodies were used at a dilution of 1:1000 (Cell Signaling Technology Inc., Beverly, MA, USA): Rabbit anti-phospho Akt (Ser473), rabbit anti-phospho Akt (Thr308), rabbit anti-Akt, rabbit anti-phospho GSK-3β (Ser9), anti-phospho FKHR (Ser256), anti-phospho PTEN (Ser380), rabbit anti-phospho PDK-1, rabbit Phospho-PKCα/βII (Thr638/641) antibody, rabbit anti-phospho-PKCδ (Thr505) antibody, rabbit anti-phospho-PKCδ (Ser643) antibody, rabbit anti-phospho-PKD/PKCμ (Ser744/748) antibody, rabbit anti-phospho-PKD/PKCμ (Ser916) antibody, rabbit anti-phospho-PKCζ (Thr538) antibody, and rabbit anti-phospho-PKCζ/λ (Thr410/403) antibody. Rabbit anti-PKCζ antibody (diluted 1:1000) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-SARS N and M antibodies were described previously (Mizutani, 2004). After 15-h incubation, the membrane was washed with 0.1% TBS-Tween and specific proteins were detected with a ProtoBlot II AP system (Promega Co., Madison, WI, USA), as described previously (Mizutani et al., 2003).

In vitro GSK-3 activation assay

Vero E6 cells were infected with SARS-CoV at moi of 10 for 18 hpi, and then cell extracts were obtained using the lysis buffer supplied with the Akt kinase assay kit (Cell Signaling Technology Inc., Bedford, MA, USA). Selective immunoprecipitation of Akt was performed using immobilized Akt (serine phosphorylation) antibody. After incubation of immunoprecipitated Akt in kinase buffer containing GSK-3β fusion protein and ATP, GSK-3α/β (Ser21/9) phosphorylation was analyzed by Western blotting using anti-phospho GSK-3 antibody.

DNA fragmentation

Vero E6 cells were pretreated with LY294002 for 1.5 h, and then 5 moi of SARS-CoV was inoculated. At 30 hpi, DNA was extracted using MEBCYTO Apoptosis ladder detection kit (Medical and Biological Laboratories, Co. LTD., Nagoya, Japan), as described previously (Mizutani et al., 2004). The DNA was analyzed by electrophoresis on 1% agarose gels and then stained with ethidium bromide.

Endocytosis assay

Vero E6 cells at 50% confluency in 24-well plates were incubated for 1.5 h with 10 or 20 μM LY294002 (Cell Signaling Technology Inc.) or dimethyl sulfoxide (DMSO) in 1 ml of medium. The LY294002 was dissolved in DMSO. The medium was removed and 5 μg/ml of acridine orange (Wako, Osaka, Japan) in Mg^{2+}-free Hank's solution was added to the cells. After incubation for 5 min, the cells were washed twice with Hank's solution, followed by addition of 0.5% trypan blue solution. Fluorescence micrographs were taken at the same shutter speed for each experiment.

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