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Porcine arterivirus activates the NF-κB pathway through IκB degradation

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Abstract

Nuclear factor-kappaB (NF-κB) is a critical regulator of innate and adaptive immune function as well as cell proliferation and survival. The present study demonstrated for the first time that a virus belonging to the Arteriviridae family activates NF-κB in MARC-145 cells and alveolar macrophages. In porcine reproductive and respiratory syndrome virus (PRRSV)-infected cells, NF-κB activation was characterized by translocation of NF-κB from the cytoplasm to the nucleus, increased DNA binding activity, and NF-κB-regulated gene expression. NF-κB activation was increased as PRRSV infection progressed and in a viral dose-dependent manner. UV-inactivation of PRRSV significantly reduced the level of NF-κB activation. Degradation of IκB protein was detected late in PRRSV infection, and overexpression of the dominant negative form of IκBα (IκBαDN) significantly suppressed NF-κB activation induced by PRRSV. However, IκBαDN did not affect viral replication and viral cytopathic effect. PRRSV infection induced oxidative stress in cells by generating reactive oxygen species (ROS), and antioxidants inhibited NF-κB DNA binding activity in PRRSV-infected cells, suggesting ROS as a mechanism by which NF-κB was activated by PRRSV infection. Moreover, NF-κB-dependent expression of matrix metalloproteinase (MMP)-2 and MMP-9 was observed in PRRSV-infected cells, an observation which implies that NF-κB activation is a biologically significant aspect of PRRSV pathogenesis. The results presented here provide a basis for understanding molecular pathways of pathology and immune evasion associated with disease caused by PRRSV.

Keywords: PRRSV; NF-κB; Porcine alveolar macrophages; Reactive oxygen species; Matrix metalloproteinase

Introduction

PRRSV is an enveloped, positive-stranded RNA virus that is a member of the order *Nidovirales*, family *Arteriviridae*, along with lactate dehydrogenase-elevating virus of mice, equine arteritis virus, and simian hemorrhagic fever virus. PRRSV causes one of the most economically important diseases of swine which is characterized by severe and sometimes fatal respiratory disease and reproductive failure. Infection with PRRSV also predisposes pigs to infection by bacterial and viral pathogens such as *Steptococcus suis*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Actinobaccillus pleuropneumoniae*, *Salmonella* spp., and swine influenza virus (Benfield et al., 1992; Done and Paton, 1995; Galina et al., 1994; Groschup et al., 1993; Kawashima et al., 1996; Zeman et al., 1993). The most consistent pathological lesions caused by PRRSV during acute infection are interstitial pneumonia and mild lymphocytic encephalitis (Halbur et al., 1995; Plagemann, 1996; Rossow et al., 1995, 1996). Tissue macrophages and monocytes are the major target cells during both acute and persistent infection (Molitor et al., 1997), although pneumocytes and epithelial germ cells of the testis have also been shown to be infected (Sur et al., 1996, 1997).

Viruses are known to control cellular signal transduction pathways, and the NF-κB pathway is a common target of many viruses. NF-κB is an inducible transcription factor that plays a key role in inflammation, innate immune responses, the regulation of cell proliferation, and cell

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survival (Caamano and Hunter, 2002; Li and Verma, 2002). Activation of NF-κB by viral infection is a key trigger to inducing type I interferon (IFN) transcription and other immune responses, including pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMPs), cyclooxygenase 2 (COX2), and inducible nitric oxide synthase (iNOS) (Caamano and Hunter, 2002; Santoro et al., 2003). These molecules are involved in initiating adaptive immune responses by recruiting immune cells to the site of infection. Furthermore, it was recently demonstrated that temporally activated NF-κB confers an essential innate antiviral response against cytoplasmic RNA viruses (human parainfluenza virus type 3 and respiratory syncytial virus) in an IFN-independent manner, showing the importance of NF-κB in the innate antiviral response (Bose et al., 2003).

NF-κB exists as a homodimer or heterodimer comprised of one or two of five subunits, RelA (p65), p50, RelB, c-Rel, and p52. The predominant form is a heterodimer composed of p50 and RelA subunits (Ghosh et al., 1998). When inactive, NF-κB is sequestered in the cytoplasm by associating with inhibitory proteins of the IκB family, including IκBα, IκBβ, and IκBε, which mask the nuclear localization signal. In response to a wide range of stress signals (e.g., lipopolysaccharide (LPS), tumor necrosis factor (TNF), interleukin (IL)-1, and virus infection), the inactive NF-κB–IκB complex is dissociated via serine phosphorylation by IκB kinase (IKK) and degradation of IκB in proteasomes. These events lead to the unmasking of the nuclear localization sequence of NF-κB, which then allows NF-κB to enter the nucleus and activate transcription of target genes (May and Ghosh, 1998).

While the immune response against PRRSV is not fully characterized, experimental work has demonstrated that the adaptive immune response of PRRSV-infected pigs is generally ineffective (Horter et al., 2002; Murtaugh et al., 2002; Wills et al., 1997, 2003). Specific evidence of an ineffective adaptive immune response includes a slow neutralizing antibody response, which is typically not detected until 3 weeks p.i. (Albina et al., 1998b) and does not reach maximum levels until 10–18 weeks p.i. (Nelson et al., 1994; Yoon et al., 1995). While the importance of a cell-mediated response for protection against PRRSV is well accepted, the effectiveness of this response during the early phases of disease also appears to be suboptimal (Murtaugh et al., 2002). For example, the T-cell response to PRRSV is weak and transient and cannot be re-stimulated for more than 4 weeks post-challenge (Molitor et al., 1997; Xiao et al., 2004). Additionally, IFN-γ responses of PRRSV-infected pigs were relatively weak and increased slowly in comparison to pseudorabies-virus-infected pigs (Meier et al., 2003). Although the precise mechanisms for the ineffective nature of the adaptive immune response to PRRSV are not known, PRRSV evasion of the innate immune responses, such as the type I IFN response, may set the stage for subsequent subversion of the adaptive immune response. Previous studies demonstrated that PRRSV appears to elicit weak innate interferon and cytokine responses compared to other viruses such as swine influenza virus (SIV), porcine respiratory coronavirus (PRCV), transmissible gastroenteritis (TGE) virus, and pseudorabies virus (Albina et al., 1998a; Meier et al., 2003; Van Reeth and Nauwynck, 2000; Van Reeth et al., 1999, 2002). Because NF-κB is important for regulation of type I IFN and

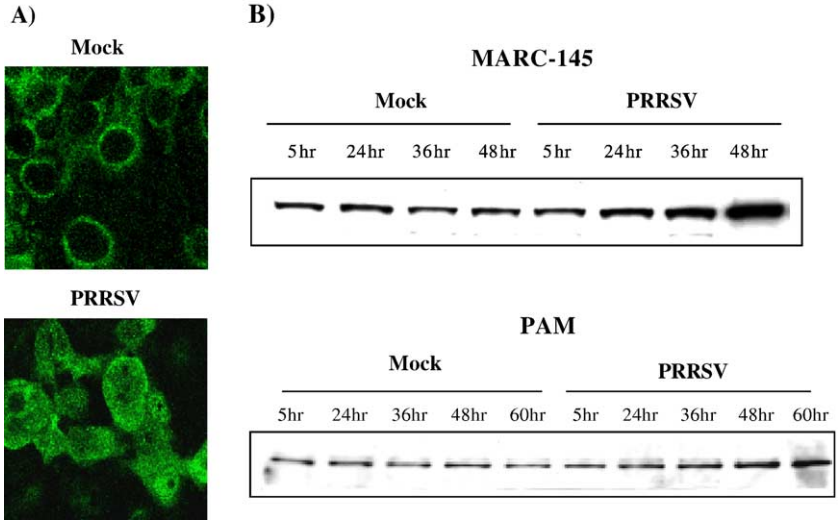

Fig. 1. NF-κB translocation is induced by PRRSV infection. (A) MARC-145 cells were infected with PRRSV at MOI = 0.1. After 48 h, cells were fixed and permeabilized followed by IFA. Nuclear translocation of NF-κB was detected by confocal laser microscopy with FITC staining following incubation with a specific antibody recognizing the NF-κB p65 subunit. (B) Nuclear translocation of NF-κB detected by Western blot of nuclear extracts from MARC-145 cells and PAM cultures. Both cell types were infected with PRRSV at MOI = 0.1. At 5, 24, 36, 48, and 60 (only in PAM) h post-infection, nuclear extracts were prepared and subjected to Western blot analysis, as described in Materials and methods.

cytokines (Lenardo et al., 1989; Mogensen and Paludan, 2001), we hypothesized that PRRSV infection may inhibit NF-κB activation to prevent antiviral responses.

The experiments presented herein were designed to determine if PRRSV infection modulates NF-κB activation in host cells. After observing NF-κB activation following PRRSV infection, the mechanisms by which PRRSV mediates NF-κB activation, as well as the role of NF-κB activation in PRRSV replication, were studied using a dominant negative form of IκBα. The present study provides a basis for understanding the molecular pathways of pathology and immune evasion associated with disease caused by PRRSV.

Results

PRRSV infection stimulates nuclear translocation and DNA binding activity of NF-κB

Nuclear translocation, which is one of the key steps during activation of NF-κB, was detected by indirect fluorescent antibody (IFA) staining following infection with PRRSV (Fig. 1A). In uninfected MARC-145 cells, p65 staining was predominantly cytoplasmic, whereas PRRSV infection (multiplicity of infection (MOI) = 0.1) for 48 h resulted in nuclear localization of NF-κB p65 staining and increased cytoplasmic staining of NF-κB p65. In Fig. 1B, Western blot analysis shows that infection with PRRSV led to accumulation of NF-κB protein in the nucleus. Compared to uninfected control cells, the amount of NF-κB increased as PRRSV infection progressed in MARC-145 cells and PAM cultures. Increased NF-κB concentrations in the nucleus were apparent at 24 h p.i. in MARC-145 cells and at 36 h p.i. in PAM cultures. In these (and subsequent) experiments, cell viability was assessed by trypan blue staining, and cultures were consistently found to be approximately 96% viable in mock-infected cells and 92% in PRRSV-infected cells at 48 h p.i.

To determine if nuclear translocated NF-κB was capable of binding κB binding motifs following PRRSV infection, an NF-κB p65 transcription factor assay was performed using nuclear extracts of MARC-145 cells and PAM cultures infected with PRRSV. MARC-145 cells and PAM cultures were either mock-infected or infected with PRRSV at MOI = 0.1, and nuclear extracts were prepared at the indicated times after virus infection. Following infection with PRRSV, NF-κB p65 DNA binding activity increased as PRRSV infection progressed. Although a slight, though statistically significant decrease was consistently observed at 24 h p.i. in PAM cultures (but not MARC-145 cells), the predominant effect of PRRSV infection in both cell types was an increase in NF-κB DNA binding activity. As shown in Fig. 2, significant increases in NF-κB DNA binding activity in PRRSV-infected cells were measured, especially at 36 and 48 h p.i, compared with that in mock-infected

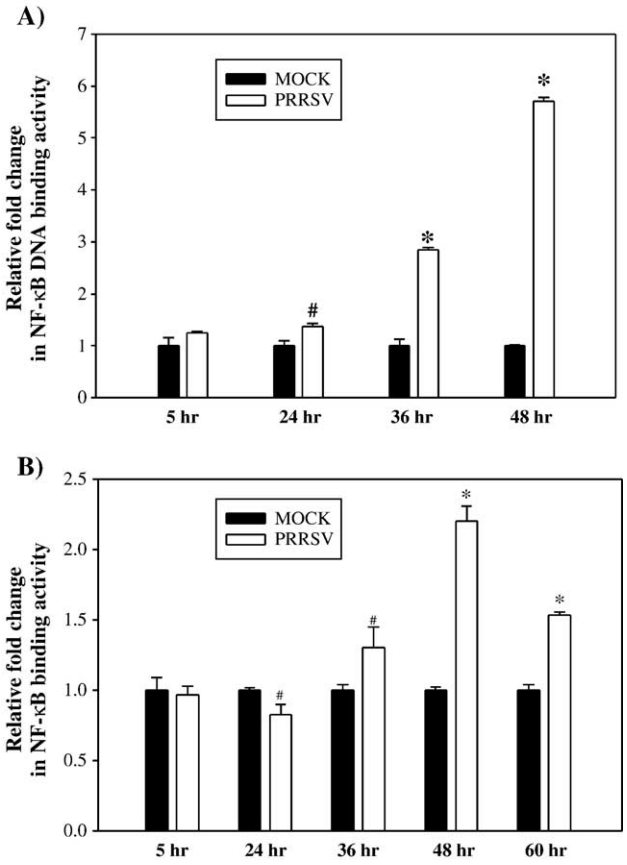

Fig. 2. NF-κB binding activity increases at the late stage of infection. NF-κB p65 binding to DNA was determined using the TransAM NF-κB p65 transcription factor assay kit. Nuclear extracts of MARC-145 cells (A) and PAM cultures (B) were prepared at 5, 24, 36, 48, and 60 (only in PAM) h post-infection. Results are expressed as the fold increase of binding activity compared to mock-infected control. These results are representative of three (MARC-145 cells) or two (PAM cultures) independent experiments. Values are shown as the mean ± SD from triplicate wells. #$P < 0.05$ compared to mock-infected control, *$P < 0.01$ compared to mock-infected control.

cells. At 48 h p.i., NF-κB p65 DNA binding activities increased 5.7-fold in MARC-145 cells and 2.2-fold in PAM cultures. The NF-κB DNA binding activity observed in these assays was ablated by an excess of unlabeled competitor, but not by an excess of unlabeled noncompetitor (data not shown). Taken together, these results demonstrated that PRRSV induces nuclear translocation of NF-κB followed by increased DNA binding activity of NF-κB both in MARC-145 cells and PAM cultures.

PRRSV enhances NF-κB-regulated gene expression, and NF-κB activation by PRRSV is dependent on viral dose and active viral replication

Translocation into the nucleus allows NF-κB to stimulate expression of target genes. Thus, an NF-κB reporter assay was used to determine if PRRSV infection enhanced NF-κB-regulated gene expression. MARC-145 cells were transiently transfected with an NF-κB luciferase reporter

plasmid (NF-κB-LUC), which contains κB binding motifs under the control of a CMV promoter. Therefore, luciferase expression is under control of NF-κB activation. After transfection, cells were infected with PRRSV for 1 h or left uninfected. NF-κB-regulated luciferase expression was significantly enhanced during PRRSV infection at 36 and 48 h p.i. which correlated with an increased level of NF-κB DNA binding activity (Fig. 3A). NF-κB activity was 2.53-fold and 6.08-fold higher in PRRSV-infected cells at 36 and 48 h p.i. compared to uninfected control cells. These findings show that PRRSV infection stimulated NF-κB-regulated gene expression late in infection, which means that NF-κB activated by PRRSV is transcriptionally active and functional.

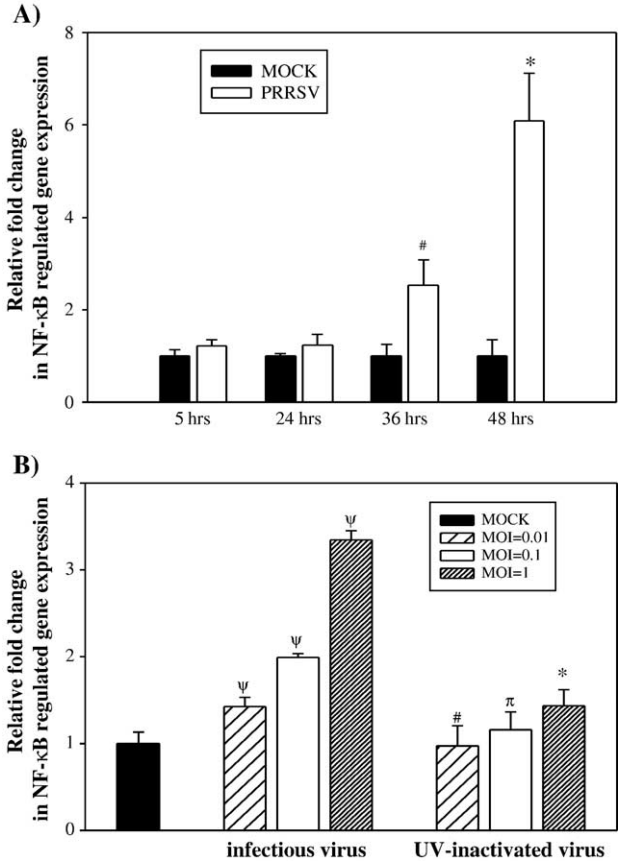

Fig. 3. PRRSV enhances NF-κB-regulated gene expression in a viral dose-dependent manner. (A) MARC-145 cells were transfected with pNF-κB-Luc and phRG-TK, then cells were either mock-infected or infected with PRRSV at MOI = 0.1. Cells were lysed at 5, 24, 36, and 48 h post-infection. #$P < 0.05$ compared to mock-infected control, *$P < 0.01$ compared to mock-infected control. Results are representative of at least three independent experiments. (B) MARC-145 cells transfected with NF-κB-Luc and phRG-TK plasmid were infected with PRRSV or UV-inactivated PRRSV at MOI = 1, 0.1, and 0.01 for 1 h. After washing with PBS, fresh medium was added. At 24 h post-infection, cells were lysed. Firefly and renillar luciferase activities were measured by using a dual luciferase reporter assay kit. The luciferase assays were performed in triplicate. Firefly luciferase activity was normalized by renillar luciferase activity. Results are shown as the relative fold change compared to that of mock-infected cells. The symbol ψ indicates $P < 0.001$ for results from infectious virus compared to mock-infected controls. The symbols #, *, and π indicate P values <0.01, <0.001, and <0.05, respectively, for results from UV-inactivated virus compared to infectious virus. These results are representative of at least three independent experiments. Each bar represents the averaged data from one representative experiment. Values are shown as the mean ± SD from triplicate wells.

To determine if there was a relationship between PRRSV replication and NF-κB activation, MARC-145 cells were infected at various MOIs and NF-κB activation was monitored by measuring NF-κB-regulated luciferase expression. As shown in Fig. 3B, a higher MOI resulted in higher levels of NF-κB activation, suggesting that NF-κB activation by PRRSV is viral dose-dependent. In some viral infections, binding of the viral particle to a cellular surface receptor is sufficient to trigger signaling cascades that activate NF-κB. To test this possibility, UV-inactivated PRRSV was used to determine if PRRSV binding to its receptor mediates NF-κB activation. As shown in Fig. 3B, UV-inactivation of PRRSV decreased NF-κB activation compared to infection with non-inactivated (fully infectious) PRRSV. NF-κB-regulated gene expression was reduced from 3.34-fold to 1.43-fold at MOI = 1, from 1.99-fold to 1.15 at MOI = 0.1, and from 1.42-fold to 0.96-fold at MOI = 0.01. These data demonstrated that NF-κB levels are elevated primarily as a result of PRRSV replication and that UV-inactivation of PRRSV significantly decreased this effect.

IκB proteins are degraded by PRRSV infection

A key step that leads to NF-κB activation in response to many extracellular stimuli is degradation of IκB proteins. Therefore, it was determined if PRRSV-mediated changes in the IκB proteins correlated with increased NF-κB activity. Protein levels of IκBα, IκBβ, and IκBε were monitored by Western blot analysis following a time course of infection in MARC-145 cells and PAM cultures. Results in Fig. 4 show that IκBα, IκBβ, and IκBε were found in uninfected MARC-145 cells and PAMs and the protein levels were mostly unchanged throughout the time course. In MARC-145 cells (Fig. 4A), PRRSV infection resulted in a lower concentration of IκBα at 48 h p.i. compared to uninfected control cells, suggesting proteosomal degradation of IκBα. This correlated with the highest NF-κB activity at 48 h p.i. among the time points tested. However, IκBα was still weakly detectable in PRRSV-infected cells at late times p.i. IκBβ and IκBε remained relatively constant at all time points in PRRSV-infected MARC-145 cells. In PAM cultures, degradation of IκBα and IκBε was detected after PRRSV infection at 48 h and 60 h p.i. as shown in Fig. 4B. The degradation of IκBβ protein was detected at 60 h p.i. The onset of the degradation of IκB proteins correlated with the NF-κB activation later in PRRSV infection as observed above. For both MARC-145 cells and PAM cultures, the same blot was also reacted with an actin-specific antibody to confirm that comparable amounts of protein were loaded in each lane.

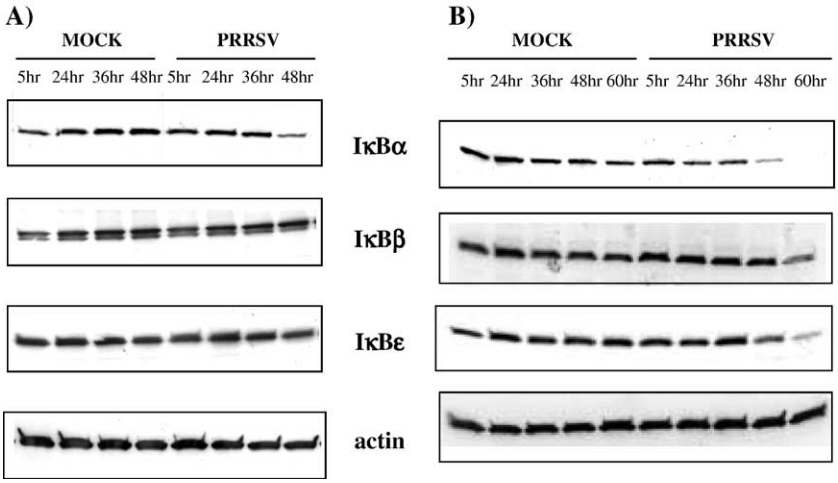

Fig. 4. IκB proteins are degraded in PRRSV-infected cells. MARC-145 cells (A) and PAM (B) cultures were infected with PRRSV at MOI = 0.1. Cytoplasmic extracts were prepared at indicated time points and subjected to Western blot analysis with antibodies specific for IκBα, IκBβ, or IκBε. Anti-actin was included as a control for sample loading. Western blot analyses were repeated in two independent experiments with similar results. A representative blot is shown.

IκBα degradation is involved in NF-κB activation by PRRSV, and blocking NF-κB activation did not affect PRRSV replication

To determine if NF-κB activation by PRRSV was dependent on IκBα degradation in MARC-145 cells, the NF-κB pathway was blocked by using an adenovirus vector expressing a dominant negative form of IκBα (Ad-IκBαDN) which lacks both constitutive (Barroga et al., 1995) and inducible (Brown et al., 1995) phosphorylation sites. Thus, IκBαDN is a potent NF-κB inhibitor. The same adenovirus vector expressing GFP (Ad-eGFP) instead of IκBαDN was used as a control. MARC-145 cells were infected with Ad-eGFP or Ad-IκBαDN at various MOIs (1, 10, or 100) for 16 h and then transfected with pNF-κB Luc plasmid and phRG-TK plasmid followed by superinfection with PRRSV at MOI = 0.1. After 48 h, cells were lysed and analyzed for luciferase activity. Overexpression of IκBαDN significantly suppressed constitutive NF-κB activity compared to that in the Ad-eGFP, and such IκBαDN expressing cells failed to activate NF-κB in response to PRRSV infection (Fig. 5).

It was then determined if NF-κB was required for efficient PRRSV replication. Cell culture medium was collected at 0, 5, 24, 36, and 48 h p.i. Production of infectious progeny virus was determined by serial 10-fold dilutions of viral stocks with 50% tissue culture infectious dose ($TCID_{50}$) titers calculated by the method of Reed and Muench (1938) on MARC-145 cells. The results representing three independent experiments are shown in Fig. 6. The kinetics of PRRSV replication were compared to that in control cells. The results showed that neither Ad-eGFP (Fig. 6A) nor Ad-IκBaDN (Fig. 6B) affected PRRSV replication in MARC-145 cells. In addition, a typical PRRSV-induced CPE was observed in both Ad-eGFP- and Ad-IκBaDN-infected cells. Therefore, this result showed that blocking the NF-κB pathway by overexpression of IκBαDN does not alter production of PRRSV progeny viruses.

Oxidative stress induced by PRRSV infection is involved in NF-κB activation

Intracellular ROS production was detected by staining with the hydrogen-peroxide-sensitive fluorescent dye DCFH-DA which is cleaved intracellularly by nonspecific

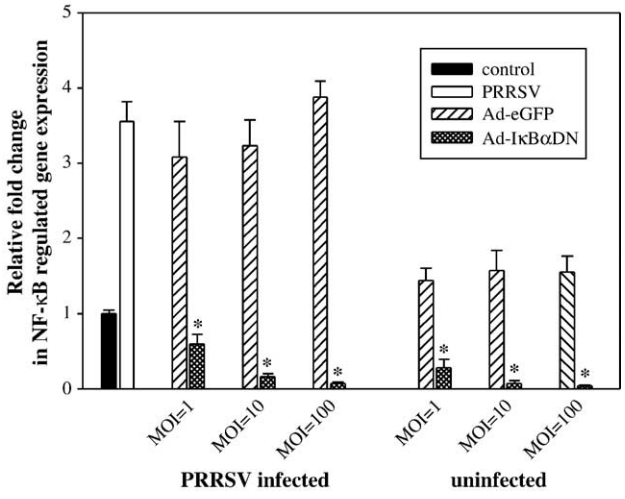

Fig. 5. Degradation of IκBα is required in NF-κB activation induced by PRRSV. MARC-145 cells were infected with Ad-eGFP or Ad-IκBαDN at different MOI for 16 h and then transfected with pNF-κB LUC plasmid and phRG-TK plasmid. Cells were then mock-infected or infected with PRRSV at MOI = 0.1. At 48 h p.i., cells were lysed, and lysates were analyzed for firefly and renillar luciferase activities using a dual luciferase reporter assay kit. Firefly luciferase activity was normalized by renillar luciferase activity. Results are shown as the relative fold change compared to that of mock-infected cells. For all assays, analysis was performed in triplicate, and values are shown as mean ± SD. These results are representative of at least three independent experiments. *$P < 0.001$.

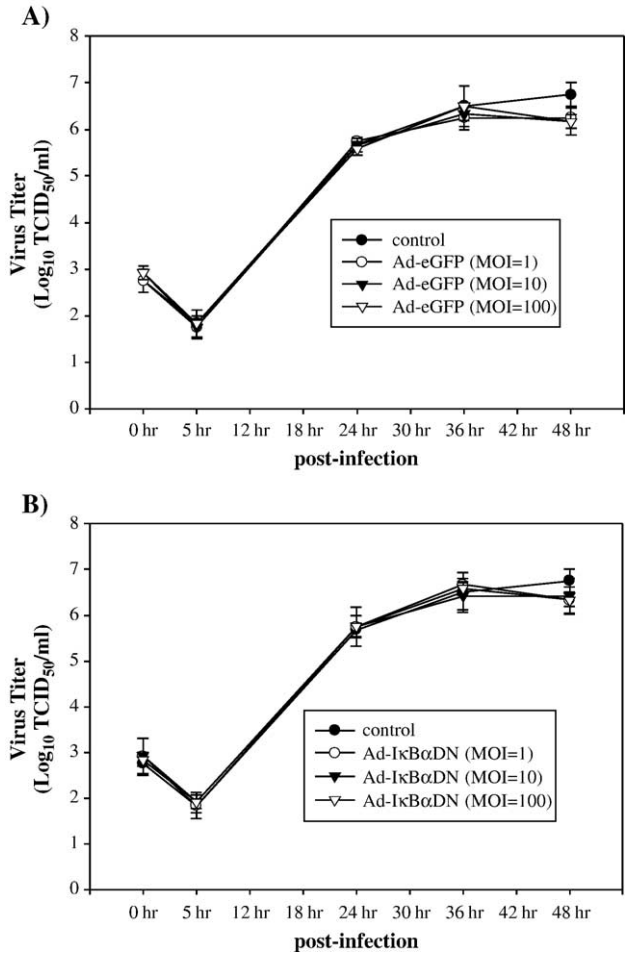

Fig. 6. NF-κB activation is not essential for PRRSV replication. MARC-145 cells were infected with Ad-eGFP (A) or Ad-IκBαDN (B) at different MOI for 16 h and then were superinfected with PRRSV at MOI = 0.1 for 1 h. Cell culture medium was collected at 0, 5, 24, 36, and 48 h p.i., with each time point represented by triplicate samples. Samples were frozen −80 °C and thawed one time, and infectious virus titers were analyzed in MARC-145 cells. Values are shown as the mean ± SD from triplicate wells, and this experiment was repeated twice with consistent results.

esterases to form DCFH. ROS in the cells then oxidizes DCFH to form the fluorescent product DCF (Sawada et al., 1996). As shown in Fig. 7A, DCF fluorescence was enhanced in cells infected with PRRSV at 48 h p.i, suggesting that PRRSV induced ROS production in MARC-145 cells. To determine if ROS induction by PRRSV contributed to activation of NF-κB, cells were treated with antioxidants and NF-κB binding activity was measured at 48 h p.i. The DNA binding activity of NF-κB p65 was markedly reduced in PRRSV-infected cells when treated with the antioxidant PDTC or NAC as shown in Fig. 7B. The highest concentration of PDTC or NAC used in this experiment reduced NF-κB activity to less than 30% in PRRSV-infected cells. To rule out that the observed effect is simply due to inhibition of viral replication by antioxidants, virus titer was determined at 24 and 48 h p.i. (Fig. 7C). PRRSV replication was not significantly affected by either PDTC or NAC in all concentration tested.

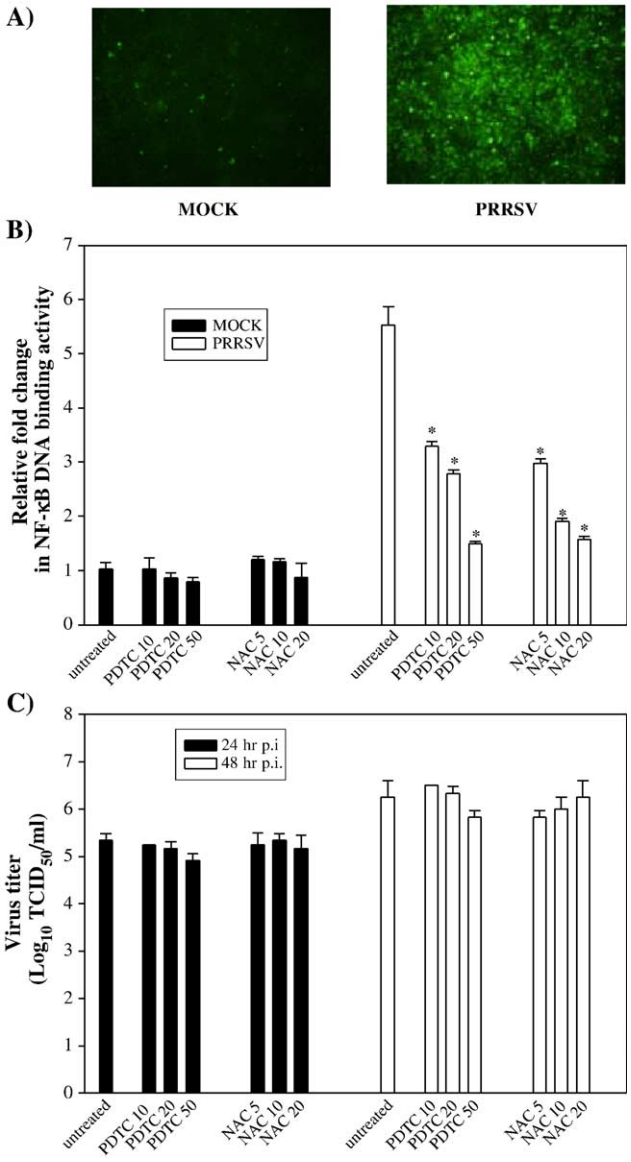

Fig. 7. ROS induction by PRRSV is involved in NF-κB activation. (A) MARC-145 cells were infected with PRRSV at MOI = 0.1 for 48 h. DCF fluorescence was measured by fluorescence microscopy. (B) MARC-145 cells were infected at MOI = 0.1 with PRRSV with or without antioxidant (PDTC or NAC). At 48 h p.i., nuclear extracts were prepared, and NF-κB p65 binding to DNA was determined using the TransAM™ NF-κB p65 transcription factor assay kit. Results are expressed as the fold increase of binding activity compared to mock-infected control. *$P < 0.001$ compared to untreated PRRSV control. (C) Cell culture medium was collected at 24 and 48 h p.i., with each time point represented by triplicate samples. Samples were frozen −80 °C and thawed one time, and infectious PRRSV titers were analyzed in MARC-145 cells. These results are representative of two independent experiments. Values are shown as the mean ± SD from triplicate samples.

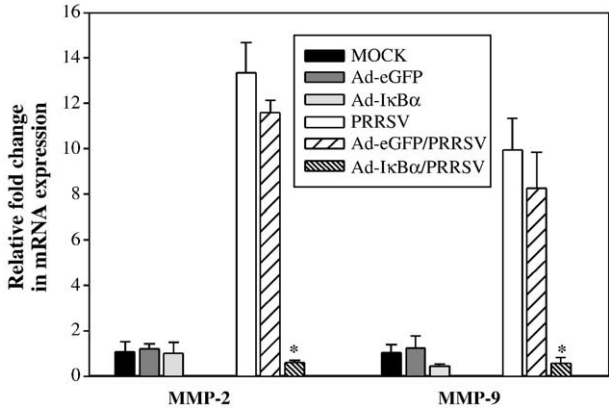

Fig. 8. PRRSV increases MMP-2 and MMP-9 gene expression through an NF-κB-dependent pathway. MARC-145 cells were infected with PRRSV for 48 h at MOI = 0.1, and total RNA was extracted and treated with DNase I. Quantitative real-time RT-PCR was performed for MMP-2 or MMP-9 specific primers. Results are expressed as relative fold changes of MMP-2 or MMP-9 mRNA using cyclophilin as an internal control. Values are shown as the means ± SD from triplicate wells and represent two independent experiments. *$P < 0.001$ compared to Ad-eGFP/PRRSV-infected cells.

MMP-2 and MMP-9 induction by PRRSV is dependent on NF-κB activation

To investigate a possible biological role of NF-κB activation in PRRSV pathogenesis, mRNA expression of MMP-2 and MMP-9 which are regulated by NF-κB were determined at 48 h p.i. As shown in Fig. 8, MMP-2 and MMP-9 gene expression were significantly enhanced by PRRSV infection which increased expression by approximately 13- and 10-fold, respectively. Overexpression of IκBαDN completely blocked MMP-2 and MMP-9 gene expression. Therefore, these data indicate that the activation of the NF-κB pathway by PRRSV was necessary for enhanced mRNA expressions of MMP-2 and MMP-9.

Discussion

Virus–host interactions lead to both activation and inhibition of complex cellular pathways, resulting in antiviral responses as well as enhanced viral replication and virulence. Despite years of research, little is known about intracellular signaling pathways that play key roles after PRRSV infection and the role of these pathways in PRRSV pathogenesis. The present study demonstrated for the first time that a virus belonging to the *Arteriviridae* family activates NF-κB in host cells and that potential mechanisms of PRRSV-mediated NF-κB activation are derived from the IκB protein degradation and ROS induction. The major target cells of PRRSV in vivo are tissue macrophages such as PAMs. MARC-145 cells are the only continuous cell line that is highly permissive for PRRSV infection (Kim et al., 1993), and they are typically used for in vitro experiments of PRRSV as well as virus maintenance and attenuation in the laboratory. Viruses could have different effects on NF-κB pathways depending on the cell type infected as demonstrated in Epstein–Barr virus and measles virus infection (Devergne et al., 1996; Dhib-Jalbut et al., 1999; Dreyfus et al., 1999; Fang et al., 2001; Helin et al., 2001). Therefore, in the present study, both MARC-145 cells and PAM cultures were used to determine if PRRSV activates the NF-κB pathway. This study showed that PRRSV infection resulted in increased nuclear translocation of NF-κB and increased DNA binding activity both in MARC-145 cells and PAM cultures. In addition, NF-κB-dependent luciferase expression was significantly increased in MARC-145 cells by PRRSV infection. Although the NF-κB reporter assay was not successfully performed in PAM cultures (due to extremely low transfection efficiencies of PAM cultures), results presented here clearly demonstrated that PRRSV activates the NF-κB pathway in both its natural target cells, PAM, as well as in a continuous cell line, MARC-145 cells.

Viruses have developed various strategies which lead to either activation or inhibition of NF-κB-dependent gene transcription for their benefits (Santoro et al., 2003). The NF-κB pathway can be activated as a protective response of the host to viruses. Therefore, some viruses, such as vaccinia virus, African swine fever virus, influenza A virus, and mengovirus, have evolved strategies to block NF-κB activation in order to evade the innate immune response (Powell et al., 1996; Shisler and Jin, 2004; Wang et al., 2000; Zoll et al., 2002). The NF-κB pathway can also be activated directly by viruses. Viruses including HIV, herpesviruses, hepatitis C virus, encephalomyocarditis virus, reovirus, dengue virus, West Nile virus, and herpes simplex virus have evolved strategies to activate NF-κB to exploit NF-κB for optimized replication, or to control host cell proliferation and survival to maximize viral progeny production (Connolly et al., 2000; Goodkin et al., 2003; Jan et al., 2000; Santoro et al., 2003; Schwarz et al., 1998; Waris et al., 2003). Despite the importance of the NF-κB pathway in immune response, it has not been determined if PRRSV or other arteriviruses modulate this pathway. Previous studies demonstrated that PRRSV induced weak type I IFN responses (Albina et al., 1998a; Lee et al., 2004; Miller et al., 2004; Van Reeth et al., 1999). Therefore, it has been postulated that PRRSV inhibited the NF-κB pathway to evade antiviral responses of host cells. However, the present study provides evidence that PRRSV actually activates the NF-κB pathway in PAMs, which are primary target cells in vivo. The synthesis of type I IFN is regulated trascriptionally and post-transcriptionally, and various transcription factors such as the interferon regulatory factor (IRF) family as well as NF-κB may be involved (Hiscott et al., 2003; Kim et al., 2000; Taniguchi and Takaoka, 2002; Wathelet et al., 1998). Therefore, it is possible that PRRSV blocks IFN gene expression at transcriptional and/or post-transcription levels but does not inhibit the NF-κB pathway.

A number of studies have suggested that oxidative stress induced by increased generation of ROS is involved in the activation of NF-κB (Ghosh and Karin, 2002; Janssen-Heininger et al., 2000). Virus infections such as human immunodeficiency virus (HIV) (Israel and Gougerot-Pocidalo, 1997), cytomegalovirus (CMV) (Speir, 2000), influenza virus (Flory et al., 2000), hepatitis B virus (HBV) (Waris et al., 2001), hepatitis C virus (HCV) (Gong et al., 2001), Japanese encephalitis virus (Lin et al., 2004), and herpes simplex virus (Mogensen et al., 2003) activate the NF-κB pathway through ROS production. Our study demonstrated that PRRSV generated ROS, and the involvement of ROS in NF-κB activation by PRRSV was demonstrated by reduced NF-κB binding activity in the presence of PDTC and NAC. It has been shown that oxidative stress induced by ROS is associated with viral pathogenesis in case of influenza virus and HIV (Peterhans, 1997; Schwarz, 1996). However, the role of ROS in PRRSV pathogenesis remains to be elucidated.

Viruses modulate NF-κB activation through various mechanisms. Activation of NF-κB is usually mediated by degradation of IκBα in a proteasome-dependent mechanism after phosphorylation by IKK (Hayden and Ghosh, 2004). IκBα is generally thought to be the major inhibitor of NF-κB activation. NF-κB activation by influenza virus is mediated by oxidative radicals and activation of IKK as a result of overexpression of viral proteins in endoplasmic reticulum (Flory et al., 2000). The Tax transactivator oncoprotein of human T-lymphotropic virus-1 activates NF-κB by interacting directly with IKK (O'Mahony et al., 2004). HSV-1 induces persistent translocation of NF-κB by IκBα degradation (Patel et al., 1998). In this study, Western blot analysis of IκB protein levels revealed that IκBα protein was degraded in PRRSV-infected cells and the expression of IκBα-DN eliminated NF-κB activation by PRRSV. This finding demonstrates that NF-κB activation by PRRSV is mediated at least in part by IκBα degradation in MARC-145 cells and the degradation of IκBα, IκBβ, and IκBε in PAM cultures. This result indicates the possibility that different molecules are involved in NF-κB activation in PAM culture infected with PRRSV compared to MARC-145 cells. However, the precise mechanism through which PRRSV influences the IκB degradation in both cells is presently unknown.

Some viruses activate the NF-κB pathway through viral protein-cellular receptor interaction. For instance, HIV gp120 and EBV gp350 activate NF-κB signaling pathway through binding to CD4 and CD21 (Bossis et al., 2002; D'Addario et al., 1999; Sugano et al., 1997). However, it is unlikely that NF-κB activation by PRRSV is triggered solely by viral binding to its cognate cellular receptor because the level of NF-κB activation by PRRSV increased as PRRSV replication progression was significantly reduced by UV-inactivation of virus. Therefore, it is possible that PRRSV replication or viral protein expression is a prerequisite for activation of the NF-κB pathway. Alternatively, a soluble factor induced by PRRSV could be responsible for the delayed NF-κB activation.

Previous studies have demonstrated a requirement for NF-κB activation in viral replication. Influenza virus infection is dependent on an active NF-κB signaling pathway (Nimmerjahn et al., 2004). Inhibition of NF-κB activation blocked influenza virus infection of susceptible cells, and cells with low NF-κB activity were poorly susceptible to influenza virus infection (Nimmerjahn et al., 2004). Efficient replication of HSV-1 is promoted by NF-κB activation through the IκB kinase-IκB-p65 pathway (Gregory et al., 2004). However, blocking the NF-κB pathway by overexpression of dominant negative forms of IκBα did not interfere with PRRSV replication, suggesting that activation of the NF-κB pathway is non-essential for efficient viral replication. Similarly, other studies with Japanese encephalitis virus or cytomegalovirus demonstrated that NF-κB activation is not required for efficient viral replication (Benedict et al., 2004; Liao et al., 2001). Although NF-κB activation does not play an essential role in PRRSV replication in vitro, it does not mean that NF-κB has no contribution to PRRSV pathogenesis in vivo. Macrophages represent an important source for a variety of soluble immune mediators, including cytokines, chemokines, MMPs, and adhesion molecules which often contain NF-κB binding sites in their promoters (Caamano and Hunter, 2002; Kim and Koh, 2000; Li and Verma, 2002; Mogensen and Paludan, 2001). The MMPs are a group of zinc- and calcium-dependent endopeptidases that degrade an extracellular matrix implicated in tissue remodeling and chronic inflammation. MMPs, especially MMP-2 and MMP-9, play a role in immune responses by promoting infiltration of inflammatory cells (Kumagai et al., 1999). A previous study demonstrated that PRRSV infection significantly increased MMP-2 and MMP-9 which correlated with the appearance of severe histological lung lesions characterized by massive lymphomononuclear cell infiltration and possible local immunosuppression in PRRSV-infected pigs. Therefore, it is possible that MMPs produced in PRRSV-infected cells by NF-κB activation could mediate the influx of new cells of the monocyte/macrophage lineage.

It is well established that NF-κB may play a pivotal role in apoptosis of virus-infected cells. The NF-κB activation by viruses could be either an anti-apoptotic response to maximize viral replication by prolonging host cell survival or pro-apoptotic response as a mechanism to increase virus spread (Bowie et al., 2004; Mi et al., 2001). In cells infected with viruses such as Sindbis virus, reovirus, or dengue virus, apoptosis is facilitated by the activation of NF-κB which was triggered by viral infection (Connolly et al., 2000; Jan et al., 2000; Lin et al., 1995, 1998). In others, activated NF-κB prevents apoptosis and prolongs cell survival (Bowie et al., 2004; Goodkin et al., 2003; Grimm et al., 2005; Su et al., 2001; Thomas et al., 2002). PRRSV has been known to induce apoptosis mostly in bystander cells in vivo (Sirinarumitr et al., 1998). It is not known yet whether the activation

of NF-κB in PRRSV infection plays an anti-apoptotic role or pro-apoptotic role.

In summary, the present study demonstrated that PRRSV activates NF-κB via IκB degradation in MARC-145 cells and PAM cultures and that NF-κB activation is not required for efficient PRRSV replication in vitro. ROS induction likely contributes to activation of the NF-κB pathway in PRRSV-infected cells. However, the detailed molecular mechanisms and viral components underlying NF-κB activation remain to be elucidated. This study also suggested a possible role of NF-κB activation in PRRSV pathogenesis by showing that PRRSV increased MMP-2 and MMP-9 mRNA expression through the NF-κB pathway. Future studies will confirm if PRRSV activates the NF-κB pathway in vivo. Understanding the role of NF-κB activation following PRRSV infection will contribute important information about the molecular pathogenesis of PRRSV infection.

Materials and methods

Cells and virus

The MARC-145 cell line, which is a clone of the African green monkey kidney cell line MA-104, and the HEK-293 cell line, which is derived from human embryonic kidney cells were cultured and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% FBS, 0.25 μg/ml fungizone, 100 U/ml penicillin, 10 μg/ml streptomycin sulfate, and 5 μg/ml gentamicin (BioWhittaker Inc., Walkersville, MD) and then held at 37 °C in a humidified 5% CO_2 incubator.

The PAM cultures were obtained by bronchoalveolar lavage of 2- to 3-week-old domestic piglets from a PRRSV negative herd. The lungs were removed from the piglets immediately after euthanizing by electrocution, and RPMI-1640 medium (Life Technologies, Grand Island, NY) was introduced through the main stem bronchi. Bronchoalveolar lavage fluid was centrifuged at 400 × *g* for 7 min. After centrifugation, cell pellets were resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.25 μg/ml fungizone, 100 U/ml penicillin, 10 μg/ml streptomycin sulfate, and 5 μg/ml gentamicin (BioWhittaker, Walkersville, MD) and plated at a density of $2–4 \times 10^5$ cells/well in a 24-well Primaria plate (Becton Dickinson and Company, Franklin Lakes, NJ). The PAM cultures were confirmed to be PRRSV-negative by RT-PCR before used in subsequent experiments. PAM cultures were incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator and washed once with complete RPMI-1640 media before use. All cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

PRRSV isolate 25544 was obtained from clinical cases submitted to the University of Missouri's Veterinary Medicine Diagnostic Laboratory. Virus stocks of PRRSV were prepared in MARC-145 cells. A low multiplicity of infection (MOI < 0.05) was used to prepare viral stocks. For virus infection, cells were initially adsorbed with virus at the indicated MOI for 1 h at 37 °C. After 1 h of adsorption, cells were gently washed with medium. At indicated time points post-infection (p.i.), culture media were harvested for the virus titration, and cells were lysed to prepare cellular extracts.

Adenovirus vectors expressing a dominant negative IκBα or green fluorescent protein (GFP) were kind gifts from Dr. Steven L. Bachenheimer (University of North Carolina, USA). Adenovirus stocks were prepared in HEK-293 cells.

NF-κB-dependent luciferase reporter assay

Subconfluent monolayers of MARC-145 cells in a 24-well plate were transfected with 10 ng of pRL-TK (Promega, Madison, WI) and 100 ng of pNF-κB*luc* (Stratagene, La Jolla, CA) using the Lipofectamine transfection reagent (Invitrogen Carlsbad, CA). The pRL-TK plasmid contains the *Renilla reniformis* (sea pansy) luciferase gene under the transcriptional control of the herpesvirus thymidine kinase promoter and constitutively expresses low levels of renillar luciferase. The pNF-κB*luc* plasmid contains the firefly luciferase gene under the transcriptional control of a synthetic promoter containing five direct repeats of the NF-κB binding element. After transfection, cells were mock-infected or infected with viruses at the indicated MOI. At various time points post-infection, cell monolayers were lysed in 150 μl of passive lysis buffer (Promega, Madison, WI) followed by cell lysate analysis for both luciferase activities by using the Dual Luciferase Reporter assay (Promega, Madison, WI). Luciferase activity was measured as relative light units (RLUs) using a luminometer (Turner BioSystems, Inc. Sunnyvale, CA). For all assays, experiments were performed in triplicate. For each experimental point, the average of the firefly luciferase activity was divided by the average of sea pansy luciferase activity to correct for differences in transfection efficiencies. The resulting ratios were used to compare the expression of the firefly luciferase gene in virus-infected cells to that present in uninfected (mock) cells.

Cellular extracts

Cytoplasmic and nuclear protein extracts from MARC-145 cells and PAM cultures were prepared with the nuclear extraction kit (Active Motif, Inc., Carlsbad, CA) according to the manufacturer's protocol. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

NF-κB p65 transcription factor assay

NF-κB binding to κB sites was assessed using the TransAM NF-κB p65 transcription factor assay kit (Active Motif, Inc., Carlsbad, CA). In this assay, an oligonucleotide

containing the NF-κB consensus site is attached to a 96-well plate. The active form of NF-κB contained in cell extracts specifically binds to this oligonucleotide and can be revealed by incubation with antibodies using enzyme-linked immunosorbent assay technology with absorbance reading. Ten microgram of nuclear proteins was analyzed for p65 binding to κB oligonucleotide according to the manufacturer's instructions. The specificity of the assay was monitored by competition with free wild-type κB consensus oligonucleotide or mutated κB consensus oligonucleotide.

Immunofluorescence microscopy

MARC-145 cells were washed three times with PBS, fixed and permeabilized in cold methanol for 10 min at 4 °C, and washed three times with PBS. Cells were incubated for 1 h at room temperature (RT) with a 1:500 dilution of primary mouse anti-NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were then washed five times in PBS followed by incubation for 1 h with the anti-mouse IgG secondary antibody conjugated with FITC (Sigma, St. Louis, MO). The cells were washed again with PBS and then examined with an Olympus 1X70 microscope fitted with a Bio-Rad MRC-600 confocal laser (Bio-Rad, Hercules, CA).

Western blotting

Western blotting was performed by utilizing a standard protocol (Davis et al., 1994). Briefly, cytoplasmic or nuclear extracts were diluted (2:1) in 2× sample buffer and boiled for 5 min. Twenty micrograms of each extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was washed with phosphate-buffered saline–Tween 20 (TPBS), blocked in a solution of TPBS containing 5% nonfat dry milk, and then washed three times. The membrane was then incubated with primary antibody overnight at 4 °C or 1 h at RT, washed three times with TPBS, and incubated with the secondary antibody horseradish peroxidase (HRP) conjugate solution for 1 h at RT. Samples were washed three times with TPBS, and then the signal was detected with the chemiluminescent protein detection system (Amersham Biosciences, Piscataway, NJ). Antibodies used for Western blot are anti-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IκBα (Cell Signaling, Beverly, MA), anti-IκBβ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IκBε (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit IgG-HRP (Amersham Biosciences, Piscataway, NJ), and anti-actin (Sigma, St. Louis, MO).

ROS detection

Cells were treated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Calbiochem, La Jolla, CA) for 15 min and washed twice with PBS. The cells were observed under a fluorescence microscope.

Antioxidants

The antioxidants used in this study were *N*-acetyl-L-cysteine (NAC; Calbiochem, La Jolla, CA) in PBS and pyrollidine dithiocarbamate (PDTC; Sigma, St. Louis, MO) in PBS. Concentrations which did not show cytotoxicity were used in this study. None of the solvents alone affected NF-κB activation in the concentrations used in this study (data not shown).

RNA extraction

Extraction of RNA was performed using Trizol (Invitrogen, Carlsbad, CA), and the Nucleospin RNA II kit (BD Biosciences Inc., Palo Alto, CA) with DNase I digestion performed directly on the spin column according to the manufacturer's instructions.

RNA quantification standards

Heterologous competitor RNA for quantification of MMP-2, MMP-9, or cyclophilin was synthesized using the respective real-time RT-PCR primer sequences in a methodology previously described (Kleiboeker, 2003). The concentration of purified competitor RNA was estimated by measuring the absorbance at 260 nm, and the purity was assessed by determining the ratio of absorbance at 260 nm to the absorbance at 280 nm. Samples were considered to be relatively pure and suitable for use as quantification standards if the ratio was $\geq$2.0. Following purification, the RNA was serially diluted in RNase-free dH_2O and stored as aliquots at −80 °C. The number of molecules of competitor RNA/μl was estimated based on the RNA concentration and the molecular weight of the transcript.

Quantitative (TaqMan) RT-PCR

Amplification of 2 μl RNA was performed using the Qiagen QuantiTect Probe RT-PCR kit (Qiagen Inc., Valencia, CA) with thermocycling, and detection was performed in a Stratagene Mx4000 (Stratagene Inc., La Jolla, CA). Samples were analyzed in triplicate. Thermocycling conditions were: 50 °C (30 min), 95 °C (15 min), followed by 40 cycles of denaturation (94 °C, 15 s) and annealing/extension (60 °C, 60 s). The primers and probe used for 5 exonuclease (TaqMan) amplification of MMP-9 were 5′-CCACCACAACATCACCTATTGG-3′ (forward), 5′-GAAGGCGCGGGCAAA-3′ (reverse), and 6-FAM-TCCAAAACTACTCGGAAGACTTGCCGC-BHQ1-3′ (probe). The primers and probe used for 5′ exonuclease (TaqMan) amplification of MMP-2 were 5′-CCGTCGCCCATCATCAA-3′ (forward), 5′-CAGGTATTGCACTGCCAACTCT-3′

(reverse), and 6-FAM-CGATGTCGCCCCCAAAACGGA-BHQ1-3 (probe). Amplification of cyclophilin was performed as previously described (Miller et al., 2004) and was used for normalization of MMP-2 and MMP-9 transcript quantities. All oligonucleotide primers were used at a final concentration of 0.3 μM, and the dual-labeled probes were used at a final concentration of 0.2 μM. All oligonucleotide primers and probes were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The relative copy numbers of MMP-2 and MMP-9 compared to cyclophilin were determined as previously described (Pfaffl, 2001).

Statistical analysis

The Student's *t* test was used for the statistical analyses. *P* values of less than 0.05 were considered statistically significant.

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