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# Activation of NF-κB contributes to production of pig-major acute protein and serum amyloid A in pigs experimentally infected with porcine circovirus type 2

Yingjun Lv, Xiujuan Zhang, Yun Sun, Shuxia Zhang *

*College of Veterinary Medicine, Nanjing Agricultural University, Weigang 1, Nanjing 210095, China*

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ABSTRACT

Acute phase proteins (APPs) have protective and regulatory roles in the inflammatory response. Previous studies indicate that APPs in serum change after pigs are infected with porcine circovirus type 2 (PCV2), but the mechanisms underlying APP production have remained unclear. In this present study, 35-day-old pigs were challenged with PCV2 and responses compared to an uninfected control group. To investigate the concentrations of APPs in serum and the activity of NF-κB in the liver, five pigs in the PCV2-infected group were euthanized at 14, 21 and 35 days post inoculation (dpi) while four pigs were sacrificed in the control group at 0, 14, 21 and 35 days, respectively. The concentrations of pig-major acute protein (Pig-MAP), C-reactive protein (CRP) and serum amyloid A (SAA) in infected animals were increased at 14 and 21 dpi, while the concentration of alpha-1 acid glycoprotein (AGP) was lower at 35 dpi, indicating that PCV2 induced the production of APPs. Moreover, the DNA binding activity of NF-κB and expression levels of NF-κB p65 subunit (NF-κB p65) from the cytoplasm to nucleus were increased at 14 and 21 dpi in the liver of infected pigs, while the phosphorylation of IκBα (p-IκBα) in the liver was also increased at 21 dpi. This demonstrated that PCV2 infection induced the activation of NF-κB. Both SAA and Pig-MAP concentrations correlated significantly with expression levels of NF-κB p65, indicating that activation of NF-κB contributes to the production of SAA and Pig-MAP.



## 1. Introduction

The acute phase response is an innate reaction towards tissue injury and pathogen invasion. Acute phase proteins (APPs) are a group of evolutionarily conserved plasma proteins that are synthesized mainly in the liver. These proteins have protective and regulatory roles in the inflammatory response and they are often used as biomarkers of diseases for diagnosis and prognosis and for monitoring response to therapy or general health (Fey and Gauldie, 1990; Salamano et al., 2008; Tsiakalos et al., 2009; Eckersall and Bell, 2010). The production of APPs in hepatocytes is controlled by a variety of cytokines that are released during the inflammatory process. IL-6- and IL-1-type cytokines that are produced mainly by macrophages and monocytes, such as IL-1α, IL-1β, IL-6, TNF-α and IFN-γ, appear to be the major regulators (Bode et al., 2012). Expression of APPs is controlled largely at the level of transcription. Transcriptional regulation of these proteins involves a variety of different regulators, such as members of the STAT family and NF-κB. NF-κB plays an important role in the transcriptional up-regulation of C-reactive protein (CRP) (Cha-Molstad et al., 2000; Agrawal et al., 2003) and serum amyloid A (SAA) (Edbrooke et al., 1991; Betts et al., 1993), and some studies also show that NF-κB can influence whether STAT proteins act as negative or positive regulators in transcriptional signaling (Zhang and Fuller, 1997; Bode et al., 2001; Hagihara et al., 2005; Quinton et al., 2009). Thus, NF-κB is considered to be very important in controlling the expression of APPs.

Porcine circovirus type 2 (PCV2), a member of the Circoviridae family, is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS), which affects weaning piglets typically at 3–15 weeks and has a morbidity rate of 5–15% (Allan et al., 1998; Segalés and Domingo, 2002). Lymphocytic depletion and an altered pattern of cytokine expression are considered to be the key characteristics of infection. In pigs suffering from PMWS, mRNA expression levels of IL-1α and IL-10 increase, while levels of IL-2, IL-8, TNF-α and IFN-γ decrease (Sipos et al., 2004; Darwich et al., 2008). Aside from effects on lymphocytes, PCV2 can also cause lesions in the liver, such as infectious hepatitis and apoptosis (Rosell et al., 2000; Resendes et al., 2011). Different species show different patterns of APP expression, and in pigs the main positive APPs are CRP, SAA, Pig-MAP, haptoglobin (Hp) and AGP. Previous studies indicate that CRP, Hp and Pig-MAP

* Corresponding author. Tel.: +86 25 8439 5316.
*E-mail address:* shuxia@njau.edu.cn (S. Zhang).

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concentrations increase in PMWS-affected pigs (Segalés et al., 2004; Stevenson et al., 2006; Grau-Roma et al., 2009). It is possible that cytokines produced by macrophages and monocytes in PMWS-affected pigs induce the production of the APPs by binding to receptors on the hepatic surface, such as IL-1R and IL-6R. However, the precise mechanisms underlying APP production remain unclear, though NF-κB is known to play an important role in the crosstalk of IL-6- and IL-1β-induced signaling that controls hepatic APP production (Bode et al., 2012). In this present study, changes of serum APP concentrations and the NF-κB signaling pathway in hepatocytes were investigated in pigs infected with PCV2. In addition, the relationships between APP expression and the NF-κB signaling pathway were studied.

## 2. Materials and methods

### 2.1. Virus

The virus isolate PCV2-SH was provided by the Key Laboratory of Animal Disease Diagnosis and Immunology at the Nanjing Agricultural University. Previously, PCV2-SH has been shown to induce PMWS (Wang et al., 2007). The PCV2-SH stock titers were $5 \times 10^5$ 50% tissue culture infective dose (TCID50)/ml, as determined by titration on PK-15 cells using an immunofluorescence assay.

### 2.2. Experimental design

All experimental procedures were undertaken according to the guidelines of the regional animal ethics committee (Commission, 2003; Government, 2008). Thirty-one 35-day-old piglets lacking antigens and antibodies against PCV2 and the reproductive and respiratory syndrome virus were divided into two groups and housed separately. Group 1 contained 16 piglets and was designated the uninfected control group, while group 2 contained 15 animals that were challenged with PCV2. After one-week acclimation to laboratory conditions, piglets in group 2 were challenged with PCV2-SH as described previously (Krakowka et al., 2001). Briefly, each pig was inoculated intra-nasally with 4 ml PCV2-SH containing a dose of $5 \times 10^5$ TCID50. Four days after challenge, pigs in both groups were injected at four sites with 0.5 mg/ml keyhole limpet hemocyanin (KLH) emulsified in incomplete Freund's adjuvant (ICFA) (1 ml per site) at each axilla and hip. In addition, an intraperitoneal (IP) injection of 10 ml thioglycollate broth (glycan) was given to stimulate peritoneal macrophage exudation into the peritoneal cavity. Three days later the piglets were injected with the same doses of KLH/ICFA and glycan. At 11 and 19 days, piglets received additional IP injections of glycan. Blood and tissue samples were collected from control pigs at 0, 14, 21 and 35 days (four pigs selected at random each time) and from PCV2-challenged pigs at 14, 21 and 35 dpi (five pigs each time). PCV2-special antibody and PCV2 antigen in serum were detected by indirect enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) respectively, using methods described previously (Wang et al., 2007).

### 2.3. Determination of APP levels in serum

Commercial ELISA kits were used to determine levels of CRP (Uscn Life Science & Technology Co., Ltd., Wuhan, China), AGP, SAA (R&B System, USA) and Pig-MAP (PigCHAMP Pro Europa S.A., Spain). All samples were tested in duplicate according to the manufacturers' instructions.

### 2.4. Extraction of nuclear and cytoplasmic proteins

Nuclear and cytoplasmic extracts were prepared from liver tissues using a NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, USA) according to the manufacturer's instructions. Protein concentration was measured using the BCA protein assay (Thermo, USA). Nuclear extracts were stored at −80 °C.

### 2.5. Electrophoretic mobility shift assays (EMSA)

EMSA was used to detect the DNA binding activity of NF-κB, which was performed with a commercial kit (Pierce, USA) according to the manufacturer's instructions. Reaction mixtures (10 μl) containing 5 μg nuclear extract were incubated in reaction buffer for 20 min at room temperature with 10 fmol of biotin-labeled double-stranded oligonucleotide probes (NF-κB p65 oligonucleotides: 5′-CAT CGG AAA TTT CCG GAA ATT TCC GGA AAT TTC CGG C-3′ and 5′-GCC GGA AAT TTC TGG AAA TTT CCG GAA ATT TCC AT G-3′). Then samples were run on a 5% non-denaturing polyacrylamide gel and transferred to a Biodyne™ B Nylon membrane (Thermo) in 0.5× Tris–Borate-EDTA buffer (TBE buffer; pH 8.3). The biotin-labeled NF-κB p65 probe was detected using an ImageQuant LAS 4000 Biomolecular Imager (GE).

### 2.6. Western-blot detection of NF-κB p65 and p-IκBα

The quantities of p65 in the nuclear protein and p-IκBα in cytoplasmic protein extracts were measured by western blot. Protein samples of 20 μg were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis. Samples were transferred to polyvinyl difluoride membranes, which were then blocked for 1 h in 5% nonfat dry milk suspended in 0.1% Tween-20 Tris-buffered saline (TTBS; pH 7.4). Membranes were incubated overnight at 4 °C with monoclonal antibodies against NF-κB p65 (Pirece), p-IκBα (Assay Designs), histone H1 (Santa Cruz Biotechnology) or glyceraldehyde-3-phosphate dehydrogenase (Abcam). Membranes were washed in TTBS, incubated with horseradish peroxidase-conjugated secondary antibody (Univ-bio), and developed using enhanced chemiluminescence reagents. Signals were captured and measured using the ImageQuant LAS 4000 Biomolecular Imager.

### 2.7. Statistical analysis

Statistical analysis was performed using SPSS Version 11.5. Differences between groups were assessed using one-way analysis of variance tests. Pearson correlation analysis used a one-tailed test. Values were considered to differ significantly when $P < 0.05$. All data are expressed as the mean ± one standard deviation (SD).

## 3. Results

### 3.1. Clinical signs and detection of PCV2-special antibody and PCV2 antigen in serum

No disease-like symptoms were observed in uninfected control pigs, but 8 days after challenge with PCV2 the rectal temperature of each pig in the infected group increased to a mean of 40.2 °C, with three animals exceeding 40.5 °C. At 14 dpi, infected pigs showed a rough hair-coat, lethargy and slow growth. At 18 dpi, superficial lymph nodes showed obvious intumesce, especially the inguinal lymph nodes, and two pigs had dyspnea. However, none of the infected pigs died before they were sampled. Levels of PCV2-specific antibody were greater at 14 dpi in the infected animals compared to day 0 and then levels increased further at 21 dpi before reducing at 35 dpi (data not shown). Viral DNA