Inflammation, Vol. 40, No. 4, August 2017 (© 2017)
DOI: 10.1007/s10753-017-0566-9

ORIGINAL ARTICLE

Regulation of iNOS-Derived ROS Generation by HSP90 and Cav-1 in Porcine Reproductive and Respiratory Syndrome Virus-Infected Swine Lung Injury

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Abstract—In the lungs, endothelial nitric oxide synthase (eNOS) is usually expressed in endothelial cells and inducible nitric oxide synthase (iNOS) is mainly expressed in alveolar macrophages and epithelial cells. Both eNOS and iNOS are involved in lung inflammation. While they play several roles in lung inflammation formation and resolution, their expression and activity are also regulated by inflammatory factors. Their expression relationship in virus infection-induced lung injury is not well addressed. In this report, we analyzed expression of both eNOS and iNOS, the production of nitric oxide (NO) and reactive oxygen species (ROS), and expression of their associated regulatory proteins, heat shock protein 90 (HSP90) and caveolin-1 (Cav-1), in a swine lung injury model induced by porcine reproductive and respiratory syndrome virus (PRRSV) infection. The combination of upregulation of iNOS and downregulation of eNOS was observed in both natural and experimental PRRSV-infected lungs, while the combination is much enhanced in natural infected lungs. While NO production is much reduced in both infections, ROS was enhanced only in natural infected lungs. Moreover, HSP90 is increased in both natural and experimental infection and less Cav-1 expressed was observed only in the natural PRRSV-infected lungs. Therefore, the increased ROS generation is likely due to the increased iNOS and its unbalanced regulation by HSP90 and Cav-1, and it also likely causes higher endothelial dysfunction in clinical PRRSV-infected lungs.

KEY WORDS: lung injury; inducible nitric oxide synthase; reactive oxygen species; heat shock protein 90; caveolin-1; endothelial nitric oxide synthase.

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INTRODUCTION

Endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) are two members of the nitric oxide synthase (NOS) family, which catalyze arginine to produce gas signaling molecules, nitric oxide (NO). While NO produced by eNOS is at level of nanomolar concentrations, in contrast, NO production by iNOS is much higher at level of micromolar concentrations [1]. In some cases, these two enzymes also produce reactive oxygen species (ROS) [2, 3]. In the lungs, eNOS is usually constantly expressed in endothelial cells and iNOS is mainly induced to express in alveolar macrophages and epithelial cells in response to a number of stimuli, including cytokines and lipopolysaccharide (LPS) [4]. Therefore, eNOS is important for maintenance of microvascular function

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including controlling endothelial permeability, and iNOS may play a role in defense against infections by alveolar macrophages [5]. In addition, both eNOS and iNOS are complicatedly involved in lung inflammation as demonstrated by genetically modulated mice including knockout or transgenetic overexpression. While eNOS knockout mice are more prone to neonatal hypoxia due to their enlarged airspace and less lung development [6], on the another hand, deficiency of eNOS prevents mice from LPS-induced lung injury [7] and overexpression of eNOS also prevents mice from ventilation-induced lung injury [8]. Different roles of iNOS were also indicated by iNOS knockout mice. iNOS knockout mice can resist to pleurisy, lung injury, and multiple organ failure [9, 10]; however, iNOS knockout mice also presented impaired resolution of lung injury [11].

As both eNOS and iNOS play several roles in lung inflammation formation and resolution, their expression and activity are also regulated by inflammatory factors. LPS stimulates iNOS expression, but shortens eNOS mRNA half-life and reduces eNOS protein level [12]. Hydrogen peroxide usually increases eNOS expression and reduces iNOS expression [13]. Regulatory proteins such as heat shock protein 90 (HSP90) and caveolin-1 (Cav-1) regulate either their expression or enzyme activity. HSP90 is able to interact with both enzymes and enhance their activity [14, 15], and Cav-1 inhibits eNOS activity by direct protein-protein interaction [16]. In addition, Cav-1 downregulates iNOS expression [1]. Moreover, there is a cross-talk between iNOS and eNOS since upregulation of iNOS was observed in eNOS knockout mice [17].

Virus infection causes lung inflammation or lung injury [18–20]; however, expression of both eNOS and iNOS in infected lungs has not been well addressed. Porcine reproductive and respiratory syndrome (PRRS), caused by PRRS virus (PRRSV) infection, presents with widespread bleeding spots and edema in infected pigs lungs [21], and lung injury caused by PRRSV infection may serve as an animal model of virus infection-induced lung inflammation and injury [22]. In this report, we analyze the expressions of both eNOS and iNOS in swine lungs with either experimental infection or natural infection. We also analyze the productions of NO and ROS and expression of HSP90 and Cav-1 in these lungs. Our results suggest that unbalanced eNOS/iNOS expression caused by virus infection may drive more ROS production and result in further endothelial dysfunction.

MATERIALS AND METHODS

Reagents

TRIzol reagent was from Invitrogen (Shanghai, China). Super Quick RT Master Mix (First Strand cDNA Synthesis Kit) was from cwbiotech (Beijing, China). SYBR Green Real-time PCR Master Mix was from TOYOBO (Beijing, China). Protease inhibitor and other chemical reagents were obtained from Sigma (Shanghai, China). RC DC Protein Assay was from Bio-Rad (Shanghai, China). HSP90 small interfering RNA (siRNA) and negative control siRNA were obtained from Genepharma (Suzhou, China). Total Nitric Oxide Detection Assay Kit and Reactive Oxygen Species Assay Kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Antibodies

Mouse monoclonal antibody (mAb) to eNOS (cat: 610296), iNOS (cat: 610599) and caveolin-1 (cat: 610406) were from BD Biosciences (New York, American). Mouse mAb to HSP90 (sc-101494) was obtained from Santa Cruz. Mouse mAb to GAPDH was from California Bioscience (Shanghai, China). Secondary Ab was from Proteintech (Wuhan, China). Super Signal West Pico was from Thermo.

Cell Cultures

Porcine alveolar macrophage (3D4/2) cells were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS (Gibco, Shanghai, China). Cells were transiently transfected with the expression vectors or siRNA according to the manufacturer's protocol of Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China). Cells were routinely detected for mycoplasma contamination and cultured at 37 °C in 5% CO_2.

Animal Experimental Design and Tissue Collection

Animal experimental design and tissue collection were processed as described [22]. For naturally infected samples, a number of normal swine lungs and PRRSV-infected swine lungs were collected and frozen for further experiments. The pigs were at age of 3–4 months and PRRSV infection was confirmed by PCR [22].

For PRRSV infection experiment, ten Yorkshire pigs (5 weeks, healthy) free of PRRSV and porcine circovirus type 2 (PCV2) were chosen. Then, pigs were

randomly divided into control ($n = 4$) and experimental group ($n = 6$). PRRSV or PBS was injected into muscle (virus strain wuh3, 10^{-5}TCID^{50}/ml, 3 ml/kg). Clinical signs such as mental state, breathing, appetite, and rectal temperature were recorded daily. Pigs were put to death 1 week later; lung tissues were removed for further experiments.

Pathogen Detection

Total RNA was extracted for pathogen detection; reverse transcription was performed with Super Quick RT Master Mix. cDNA were used to detect PRRSV or PCV2. Primer pairs used for PCR test were same as described [22]. Then, the clinical samples were divided into control (free of both PRRSV and PCV2, $n = 4$) and PRRSV positive ($n = 6$) group according to PCR results. The experimental infection samples were detected as mentioned above.

Western Blot

Lung tissues were homogenized in lysis buffer supplemented with protease inhibitor, then put the samples on ice for 20 min, and centrifuged at 12000*g* for 20 min. The soluble protein samples were obtained for Western blot analysis. Soluble samples were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane. After blocking with 5% skim milk in TBST for 1 h at room temperature, the membranes were incubated and overnight shaking at 4 °C with Abs against iNOS, eNOS, HSP90, Cav-1, and GAPDH (diluted 1:1000). Next, the membranes were incubated with HRP-labeled secondary Ab for 1 h at room temperature. After Ab incubation steps, membranes were washed with TBST. Signals were detected by enhanced ECL detection reagents, bands were acquired by Tanon-5200 Chemiluminescent Imaging System, and densitometric analysis was obtained using Gel Pro Analyzer software.

Total NO Production Assay

Lung tissues were washed in physiological saline and homogenized in physiological saline. After centrifugation at 4000*g* for 20 min, the supernatants were obtained for protein concentration measurement and NO detection. Total NO production in lung tissue supernatants was determined by detecting nitrate and nitrite concentration according to the manufacturer's instructions of Total Nitric Oxide Assay Kit [23–25].

ROS Assay

Lung tissues were homogenized in phosphate buffer. After centrifugation at 4000*g* for 15 min, the supernatants were obtained for ROS detection. The level of ROS in supernatants was detected according to the manufacturer's instructions of ROS assay kit [26–28]. Lung tissue supernatant was incubated with DCFH-DA at room temperature for 30 min. Next, fluorescence intensity was detected at 488 nm (exCitation wavelength) and 530 nm (emission wavelength).

Statistical Analysis

Data are expressed as mean ± SEM unless other statement. Statistical differences were performed using GraphPad Prism version 5.0 software. Mean values were compared using two-tailed Student's *t* test, and results were considered significant at *P* values less than 0.05.

RESULTS

iNOS Protein Expression in Clinical and Experimental PRRSV-Infected Swine Lung

iNOS is induced in many cell types when exposed to LPS or other agents. The expression of iNOS may be influenced when swine was subjected to bacteria or virus. So we detected the change of iNOS protein expression in PRRSV-infected lung tissue. In the samples of control and PRRSV inoculation, we found that protein expression of iNOS significantly upregulated in virus-infected swine. iNOS protein expression showed a 2.5-fold increase (Fig. 1a) in virus-inoculated lungs, compared with the control. The clinical samples showed the similar results that the lungs from PRRSV-positive group displayed a 10-fold increase of iNOS protein expression (Fig. 1b). It demonstrated that higher iNOS expression might play an important role in NO production in tested samples.

Downregulation of eNOS Expression in Clinical and Experimental PRRSV-Infected Swine Lung

The expression of eNOS is also associated with NO production. To understand the role of eNOS in clinical and experimental PRRSV-infected swine lung, we examined the protein expressed in PRRSV-infected lung tissues. We found that expression of eNOS significantly decreased in

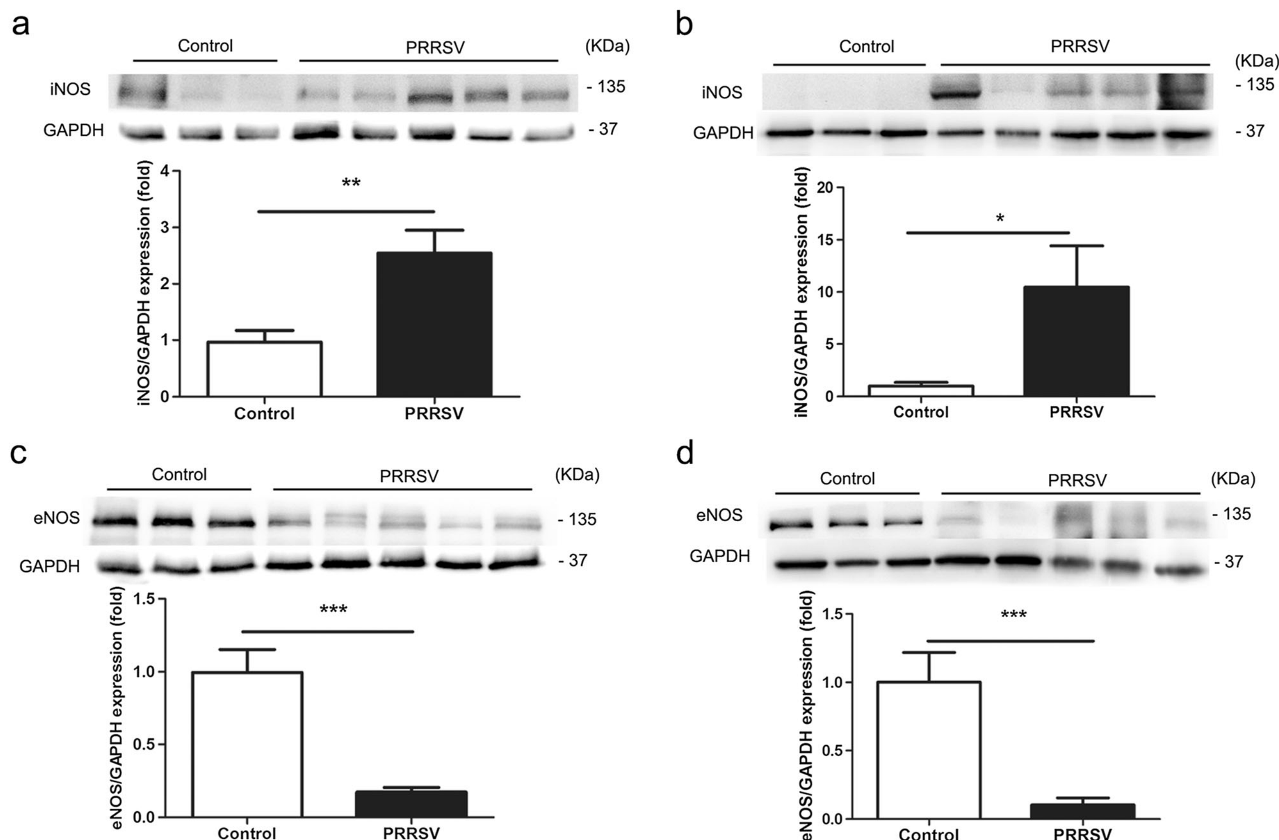

Fig. 1. iNOS and eNOS protein expression in PRRSV-infected swine lungs. Protein expression was determined by Western blot. **a**, **c** The results of samples from experimental infection. **b**, **d** The results from clinical samples. All experiments were performed 3 times for each sample. Statistical differences were determined by Student's *t* test, ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

PRRSV-infected swine. eNOS expression showed a 5.7-fold decrease (Fig. 1c) in PRRSV-inoculated lungs compared with the control. Similar result was found in clinical samples that eNOS showed a 10-fold decrease in lungs from PRRSV-positive group (Fig. 1d).

NO Production in Clinical and Experimental PRRSV-Infected Swine Lung

Superoxide interacting with NO produces peroxynitrite ($ONOO^{-}$), and the imbalance of NO and ROS generation promote endothelial dysfunction [29]. So NO production in PRRSV-infected lung samples was examined. Unexpectedly, NO production decreased 52% in PRRSV inoculation lung when compared with the control (Fig. 2a). And similar result was obtained that the concentration of nitrite and nitrate in clinical PRRSV-infected swine lung also reduced 82% (Fig. 2b). This result did not conform to increased iNOS expression. So the following experiments were performed.

ROS Generation in Clinical and Experimental PRRSV-Infected Swine Lung

High concentration of NO was usually scavenged to generate ROS in disease conditions, so excessive ROS generation often occurred in inflammatory response [30]. So we examined ROS production in PRRSV-infected lung samples. The data showed that the concentrations of ROS in the experimental PRRSV infection group had no significant change when compared with control group (Fig. 2c), while ROS concentration increased 1.5-fold in clinical PRRSV-infected swine lung compared with control (Fig. 2d).

The Expression of Cav-1 in Clinical and Experimental PRRSV-Infected Swine Lung

iNOS and eNOS are both testified binding to Cav-1, this association would inhibit the activity of eNOS and iNOS [31, 32]. So increasing Cav-1 expression may attenuate NO catalytic activity. In consideration

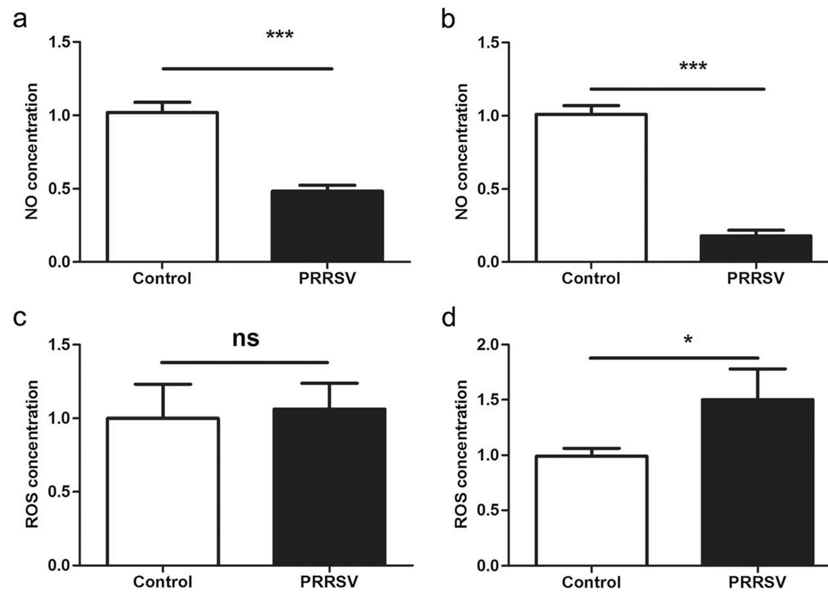

Fig. 2. NO and ROS production in PRRSV-infected swine lungs. NO and ROS production were measured according to the manufacturer's instructions of total NO and ROS assay kit. **a**, **c** The results of experimental samples. **b**, **d** The results from clinical samples. All experiments were performed 3 times for each sample. Statistical differences were determined by Student's *t* test, $*P < 0.05$; $***P < 0.001$.

of the relationship between Cav-1 and NOS-derived NO production, the expression of Cav-1 was detected in PRRSV-infected swine lung. Western blot data showed that Cav-1 protein expression presented no significant change between experimental PRRSV-infected lung and control groups (Fig. 3a). But the expression of Cav-1 in clinical samples displayed a 5.6-fold decrease to control (Fig. 3b). It suggested that higher Cav-1 expression might regulate NO bioactivity in clinical PRRSV lung but not in experimental PRRSV-infected lung.

HSP90 Expression in Clinical and Experimental PRRSV-Infected Swine Lung

HSP90 was proved as an upregulated modulator of eNOS. Also, HSP90 interacts with iNOS and enhances iNOS activity [33]. Next, HSP90 expression in PRRSV-infected swine lung was detected. Western blot data shows that HSP90 protein expression showed a 3.8-fold increase in experimental PRRSV-infected lung (Fig. 3c). Similarly, the expression of HSP90 in clinical samples displayed an 8.5-fold increase, higher than experimental PRRSV-infected lung (Fig. 3d). It suggested that higher HSP90 expression enhanced iNOS activity and may regulate ROS production.

ROS Production and iNOS Protein Expression in PRRSV-Infected Alveolar Macrophage Cells

To confirm that both Cav-1 and HSP90 are able to regulate iNOS-mediated ROS production in the infected lungs, we examined iNOS expression and ROS production in porcine alveolar macrophage cells 3D4/2 transfected with either Cav-1 cDNA or HSP90 siRNA. Cav-1 overexpression in 3D4/2 cells showed a little or no change of iNOS expression; PRRSV infection-induced ROS production in cells with Cav-1 overexpression was inhibited (Fig. 4). In the cells with HSP90 knockdown by siRNA, iNOS expression was enhanced by PRRSV infection. However, both basal and infection-induced ROS was greatly inhibited (Fig. 5). These results together suggest that both Cav-1 and HSP90 are able to regulate ROS production by iNOS at least in porcine alveolar macrophages.

DISCUSSION

Lung inflammation caused by PRRSV infection is characterized by respiratory difficulties in infected pigs. Activated neutrophils recruited into lung and generated a large amount of superoxide after PRRSV infection [34]. Studies have shown that both iNOS and eNOS are NO

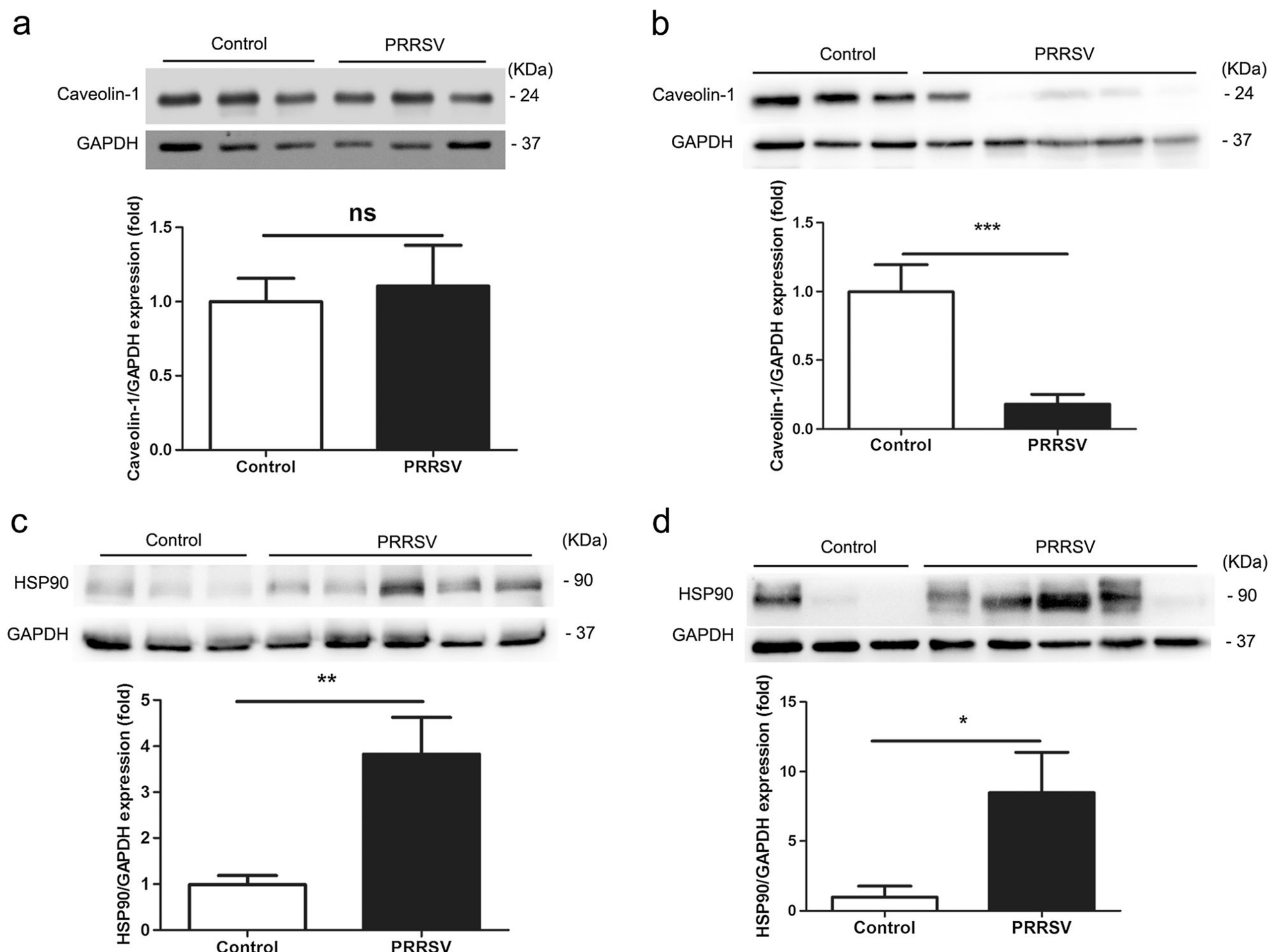

Fig. 3. Expression of Cav-1 and HSP90 in PRRSV-infected swine lungs. Protein expression was determined with Western blot analysis. **a**, **c** The results of experimental samples. **b**, **d** The results from clinical samples. All experiments were performed 3 times for each sample. Statistical differences were determined by Student's *t* test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

generator in inflammatory states [35]. However, the effects of PRRSV infection on expression of eNOS and iNOS at protein levels have not been analyzed before.

Our results from either experimental infection or natural infection clearly indicate that PRRSV infection causes downregulation of eNOS and upregulation of iNOS. The unbalanced eNOS/iNOS expression was even more dramatic in the natural infection as the ratio of iNOS/eNOS was even much higher. These results also indicated that the main NO generator is iNOS, other than eNOS, in these lungs.

We also examined the production of NO and ROS. High expression of iNOS did not result in NO production in the infected swine lungs; NO production decreased even when iNOS expression is significantly increasing (Figs. 1b and 2b). In contrast, we observed an increase ROS generation only in naturally PRRSV-infected lungs (Fig. 2d). So the decreased NO production may associate with superoxide overproduction [36, 37]. Since enhanced ROS generation caused endothelial dysfunction and tissue injury [38], it is likely that ROS may contribute to lung injury in PRRSV-infected lungs. It was reported before that highly pathogenic PRRSV infection increases ROS production [39]. Like PRRSV infection, other virus infection causing oxidative stress has been reported too. For instance, influenza virus infection also increases reactive peroxynitrite formation and causes oxidative stress in lungs [40, 41], and hepatitis C virus infection also enhances ROS generation in cells and mice [42]. Therefore, increased ROS generation may be the common characteristic for virus infection-

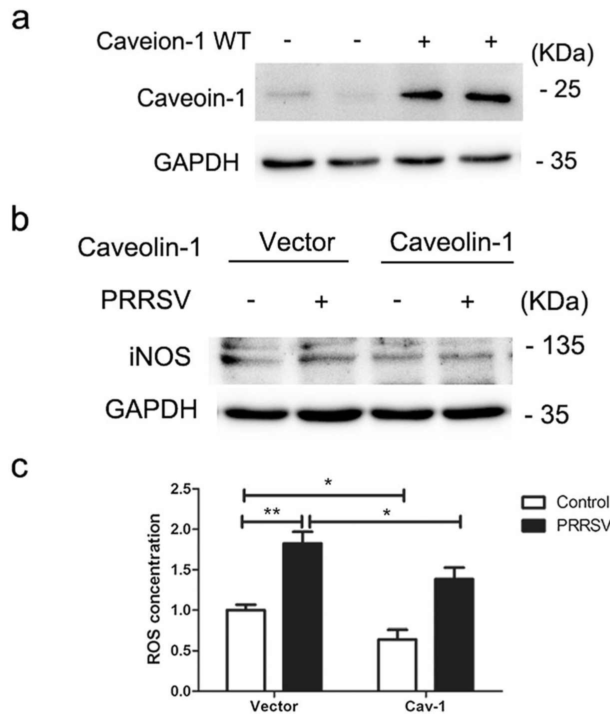

Fig. 4. iNOS protein expression and ROS production in porcine alveolar macrophage. 3D4/2 cells with Cav-1-overexpression. The 3D4/2 cells were transfected with vector or WT Cav-1 cDNA for 48 h, and PRRSV infection was conducted after transfection 24 h later. Expression of Cav-1 (**a**) and iNOS (**b**) were determined by Western blot. ROS production (**c**) was measured in 3 independent transfections. Statistical differences were determined by Student's *t* test, $^{**}P < 0.01$; $^{*}P < 0.05$.

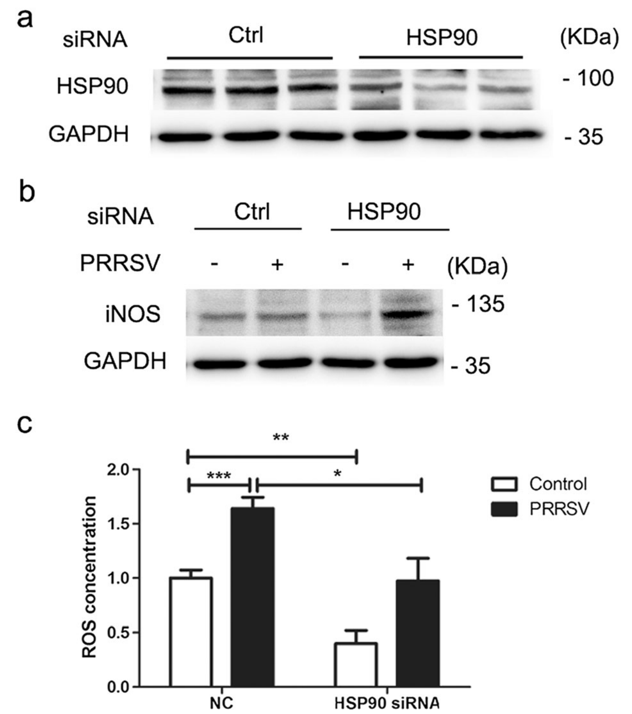

Fig. 5. iNOS protein expression and ROS production in porcine alveolar macrophage. 3D4/2 cells with HSP90 knockdown. The 3D4/2 cells were transfected with control or HSP90 siRNA for 48 h, and PRRSV infection was conducted after transfection 24 h later. Expression of HSP90 (**a**) and iNOS (**b**) were determined by Western blot, and ROS production (**c**) was measured in 3 independent transfections. Statistical differences were determined by Student's *t* test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

induced lung injury. ROS production may lead to worse endothelial dysfunction as indication of less eNOS expression in lungs compared with natural infection (Fig. 6). Suppression of ROS production can effectively weaken virus-induced lung injury [41]; understanding the regulation of ROS production will provide new view in lung injury and benefit to the future therapeutic strategy.

There are many factors regulating NOS activity. It is known that both calcium-dependent and calcium-independent signaling pathways for increasing NO production by eNOS [43–45]. And NOS activity is also regulated by HSP90 and Cav-1 [31, 32]. HSP90 enhances iNOS activity, thus increasing NO production. Our data showed higher HSP90 expression and increased ROS production in clinical PRRSV-infected lung tissues (Fig. 3d and 2d). The increased ROS production in these samples may partially be caused by HSP90 higher expression. In addition, Cav-1 reduces iNOS protein expression and activity [46]. Our data showed that Cav-1 expression decreased only in clinical samples, so decreased Cav-1 expression may also promote ROS production and inflammatory response during lung injury caused by virus infection. Our *in vitro* cellular results also supported the notion that PRRSV infection-induced ROS production by iNOS is regulated by both Cav-1 and HSP90 (Figs. 4 and 5).

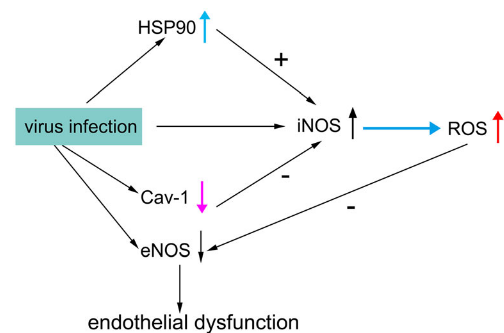

Fig. 6. Regulation of iNOS-derived ROS generation by HSP90 and Cav-1 in PRRSV-infected swine lung injury. Virus infection induces decreased eNOS expression and increased iNOS expression. Higher HSP90 expression and decreased Cav-1 expression regulates iNOS activity, thus leading to enhance ROS production, and the increased ROS production induces endothelial dysfunction in natural PRRSV-infected swine lungs.

In summary, our studies indicate that iNOS is the primary source of ROS formation in PRRSV-infected swine lung tissue, and enhanced ROS generation may play a crucial role in PRRSV-infected lung injury. Higher HSP90 expression and decreased Cav-1 expression may be two main regulatory factors for higher ROS production in natural PRRSV-infected swine lungs.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (NSFC) Grants 31372418 (G. Liu) and 31201913 (S. Zhang), NSFC Major International Cooperation Project 31210103917 (B. Liu), Huazhong Agricultural University Scientific and Technology Self-Innovation Foundation Program 2012RC011 (G. Liu), and the 948 Project of Chinese Ministry of Agriculture (2015-Z33, GL).

COMPLIANCE WITH ETHICAL STANDARDS

Animal experiments were approved by Animal Care and Use Committee of Hubei Province, China, in accordance with guidelines developed by the China Council on Animal Care and protocol.

Conflict of Interest. The authors declare that they have no conflict of interest.

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