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# *CYP1A1* mediates the suppression of major inflammatory cytokines in pulmonary alveolar macrophage (PAM) cell lines caused by *Mycoplasma hyponeumoniae*

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ABSTRACT

Mycoplasmal pneumonia is a lung infection disease caused by *Mycoplasma hyopneumoniae* in swine. We previously reported that Cytochrome P450 1A1 (*CYP1A1*) expression was significantly downregulated in pigs infected with *M. hyopneumoniae* compared to the healthy controls. In this study, pulmonary alveolar macrophage (PAM) cell lines with *CYP1A1* overexpression or siRNA-mediated *CYP1A1* silencing were used to explore the biological function and regulatory mechanism of *CYP1A1* gene expression changed on the inflammatory response of pigs infected with *M. hyopneumoniae*. The results showed that the cells overexpressing *CYP1A1* infected with *M. hyopneumoniae* led to a rapid increase in *PPAR-γ* expression, which resulted in decreasing the levels of several inflammatory cytokines including *IL-1β*, *IL-6*, *IL-8* and *TNF-α*. On the contrary, this effect was just opposite in *CYP1A1*-RNAi cells infected with *M. hyopneumoniae*. We suggest that *CYP1A1* suppress the inflammatory response caused by *M. hyopneumoniae* infection, via *PPAR-γ* signaling pathway in pigs.



## 1. Introduction

Mycoplasmal pneumonia of swine (MPS) is a chronic infectious respiratory disease caused by *Mycoplasma hyopneumoniae (M. hyopneumoniae)*. MPS is characterized by high morbidity (38%–100%) resulting in significant economic losses in pork-producing areas worldwide (Maes et al., 2008; Simionatto et al., 2013). Up to now, precise molecular mechanism for the initiation of MPS has not been elucidated. However, in previous research work we found that there were significant differences in immune response to *M. hyopneumoniae* between Chinese native pigs and Western pig breeds (Fang et al., 2015). And Chinese native pig breeds, especially Meishan and Erhualian pigs, have a higher antibody level and a more intense immune response after *M. hyopneumoniae* infection compared with the Western breeds such as the Landrace. Further studies have revealed that Cytochrome P450 enzymes may play an important role in the inflammatory response of *M. hyopneumoniae* infection in pigs (Fang et al., 2015).

Cytochrome P450 enzymes have a primary role in the metabolism of xenobiotics and a wide range of endogenous compounds. And cytochrome P450 enzymes responsible for drug metabolism have been extensively studied in humans and other animals (Danielson, 2002; Watkins, 1990; Zuber et al., 2002). Moreover, cytochrome P450s have widely varying biological functions, which are species-, sex- and tissue-dependent (Morgan et al., 1994; Ryan and Levin, 1993). Importantly, cytochrome P450 activity has been shown to be involved in the inflammatory response (Morgan, 1997; Projean et al., 2005). It has been described that, in human beings and model animals such as mouse and rabbit, the expression of different subfamilies of hepatic cytochrome P450 enzymes could be regulated by inflammation (Bleau et al., 2003; Morgan, 1997; Sewer et al., 1997), possibly leading to alter in the pharmacokinetics of xenobiotics (Morgan, 2009; Paszti-Gere et al., 2014). However, the exact mechanisms of the involved signal transduction pathways have not been clarified yet.

We previously found that Cytochrome P450 1A1 (*CYP1A1*) expression was significantly downregulated in pigs infected with *M. hyopneumoniae* compared with the naive controls (Fang et al.,

*Abbreviations: M. hyopneumoniae*, *Mycoplasma hyopneumoniae*; *CYP1A1*, cytochrome P450 1A1; PAM, pulmonary alveolar macrophage; MPS, mycoplasmal pneumonia of swine.

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2015). Pulmonary alveolar macrophage (PAM) plays a prominent role in the lung inflammatory responses caused by *M. hyopneumoniae* (Damte et al., 2011). Then, PAM cell lines were selected and used in present study to clarify whether the change in *CYP1A1* gene expression would play an important role in the inflammatory responses of *M. hyopneumoniae* infection in pigs. We established PAM cell lines with *CYP1A1* overexpression or siRNA-mediated *CYP1A1* silencing, and the experimental model for *M. hyopneumoniae* infections to explore the relationship between *CYP1A1* expression and the inflammatory response of MPS.

## 2. Materials and methods

### 2.1. Experimental pigs, PAM cells and M. hyopneumoniae strain

Meishan pigs used in the study were derived from the Shanghai Pig Breeding Farm, China. The PAM 3D4/21 cell line (ATCC CRL-2843) (Bai et al., 2013) and *M. hyopneumoniae* strain JS were donated by the Key Laboratory of Animal Disease Diagnose of Chinese Ministry of Agriculture, Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China. The Institutional Animal Ethics Committee approved this study in accordance with the national guidelines.

### 2.2. Cloning of CYP1A1 and construction of a eukaryotic expression vector

Total RNA was isolated from swine lung using Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA was reversed using the Superscript first-strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. The primer pairs for *CYP1A1* gene was designed with primer express software v.2.0.0 according to its mRNA sequence available in GenBank (No. NM_214412) (listed in Table 1). The cDNA sequence of *CYP1A1* was amplified and cloned into plasmid PMD18-T vector (TAKARA, Otsu, Shiga, Japan) to obtain a recombinant plasmid PMD18-T-*CYP1A1*, which was verified by DNA sequencing. PMD18-T-*CYP1A1* recombinant plasmid and pcDNA3.1 (+)/zeo vector (Invitrogen, Carlsbad, CA, USA) were digested with *Xho*I and *Not*I (Takara, Dalian, Co., Ltd., China), respectively, and the products were ligated using T4 DNA ligase to generate a pcDNA3.1 (+)/zeo-*CYP1A1* plasmid, which was verified by restriction enzyme digestion and DNA sequencing.

### 2.3. CYP1A1 overexpression and knockdown in PAM cells

The 3D4/21 PAM cells were treated with zeocin under a gradient concentration (100 μg/ml, 150 μg/ml, 200 μg/ml, 250 μg/ml and 300 μg/ml) to determine the optimum concentration that the cells were completely destroyed in 10–14 days.

When 80% 3D4/21 PAM cell monolayer was achieved, the cells were transfected with recombinant plasmid pcDNA3.1 (+)/zeo-*CYP1A1* using Lipofectamine 2000, and maintained in RPMI 1640 (Gibco/BRL, Grand Island, NY, USA) medium for 24 h, at 37 °C and 5% $CO_2$. This culture was screened with zeocin (using the previously established optimum concentration) for 28 days, and positive clones were preserved and amplified. Expression of *CYP1A1* was analyzed by RT-PCR and western blotting as described elsewhere (Kojima and Sekimoto, 2010), including three independent repeats in experimental and control group cells, respectively.

Furthermore, the original 3D4/21 cell line were plated in 6-well plates at a density of $2 \times 10^5$ and transfected with the *CYP1A1*-siRNA (75 pmol/well) (experiment group) or the negative control siRNA (negative control group) using Lipofectamine 2000, and maintained in RPMI 1640 (Gibco/BRL, Grand Island, NY, USA) medium for 24 h, at 37 °C and 5% $CO_2$. The *CYP1A1* siRNA (sence: 5′-GGAUGGACGAGAAUGCCAAdTdT-3′, anti-sence:5′-UUGGCAUUCUCGUCCAUCCdTdT-3′) and negative control siRNA (sence:5′-UUCUCCGAACGUGUCACGUdTdT-3′, anti-sence:5′- ACGUGACACGUUCGGAGAAdTdT-3′) were supplied by Shanghai GenePharma (Shanghai, China). qRT-PCR was used to estimate *CYP1A*1 interfering effect.

### 2.4. Artificial infection with M. hyopneumoniae

The PAM cells, including untransfected 3D4/21 cell line (normal control gruop), pcDNA3.1 (+)/zeo transfected cells (overexpression control group), pcDNA3.1 (+)/zeo-*CYP1A1* transfected cells (overexpression experimental group), *CYP1A1*-siRNA transfected cells (siRNA experiment group) and negative control siRNA transfected cells (siRNA negative control group), were plated in 6-well plates in RPMI 1640 medium supplemented with 10% fetal bovine serum at the density of $2 \times 10^5$ cells/well, respectively. The culture solution was discarded and phosphate buffer saline (PBS) was added to clean the cells two times, at the viability of PAMs was over 80%. Then the PAM cells were treated with *M. hyopneumoniae* ($10^6$ CCU/mL of live *M. hyopneumoniae* strain JS) across a time course of 0 h, 4 h, 12 h, 24 h and 48 h, respectively. RNA was extracted from the PAMs at the completion of each time point, and cDNA was synthesized from RNA as described in section 2.2. Then the infection effect was detected by PCR amplification for P36 gene, which is the special membrane protein of *M. hyopneumoniae* pathogen, as the previously described (HU et al., 2004). All of 5 groups of PAMs were cultured and processed in the same way. The experiments were independently repeated three times.

### 2.5. Detection of pro-inflammatory cytokine expression levels

Primers for pro-inflammatory cytokines (*IL-1β*, *IL-6*, *IL-8*, *TNF-α*) and *PPAR-γ* were listed in Table 1. Real-time PCR analysis was performed on an ABI 7500 detection system (Applied Biosystems, Foster City, CA) using FastStart Universal SYBR Green Master (Roche) according to the manufacturer's instructions, to quantify

**Table 1**
Primers used for real-time quantitative PCR.

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) | Fragment size (bp) |
|---|---|---|---|
| *CYP1A1* | *ATTT*GCGGCCGCATGTTCTCTGTGTTTGGAC | *CCG*CTCGAGCTAAGAGCGCACATGCA | 1572[a] |
| *IL-1β* | ACCCAAAACCTTGACCTC | GACGGATGAAAAGATGCT | 180 |
| *IL-6* | GGCTACTGCCTTCCCTAC | TACCTCCTTGCTGTTTTCAC | 187 |
| *IL-8* | GGACCAGAGCCAGGAAGA | ATTTGGGGTGGAAAGGTG | 188 |
| *TNF-α* | GCCACCACGCTCTTCTGC | GCTGTCCCTCGGCTTTGA | 172 |
| *PPAR-γ* | GCTGACCAAAGCAAAGGC | CTCCACGGAGCGAAACTG | 193 |

[a] *CYP1A1* primer includes 21 bp restriction enzyme sites and protective bases. Forward primer had 4 protective bases (italic: ATTT) and *Not*I restriction site (underline bases: GCGGCCGC). Reverse primer was 3 protective bases (italic: CCG) and *Xho*I restriction site (underline bases: CTCGAG). The length of 1551 bp *CYP1A1* gene cDNA sequence was cloned in this PCR.