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Effect of Chronic Adrenocorticotropine Hormone Treatment on LPS-induced Slight Inflammation via Inhibiting Nuclear Translocation of Glucocorticoid-glucocorticoid Receptor Complex in Lung of Pig

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Key words
Stress, Toll-like receptors, Glucocorticoid, Adrenocorticotropine, lipopolysaccharide, Pig

ABSTRACT

The crosstalk between glucocorticoid (GC) and immune response is critical for the maintenance of homeostasis. However, it remains elusive whether chronic stress affects lipopolysaccharide (LPS) -induced inflammation via the crosstalk. Pigs were administered LPS injection with or without continuous adrenocorticotropine hormone (ACTH) pretreatment. Twenty-four 30-day-old Duroc × Landrace × Large White crossbred piglets (12 ± 0.5 kg) were randomly assigned to 4 treatments: control, LPS, ACTH, and ACTH+LPS groups. Each group consisted of 6 male piglets. All ACTH pigs and ACTH+LPS pigs were injected intramuscularly with ACTH (2.25 IU/kg body weight) for 7 consecutive injections at 6-h intervals. Then, LPS pigs and ACTH+LPS pigs were injected intramuscularly with LPS (15 μg/kg body weight). LPS upregulated levels of pulmonary IL-1β, IL-10, TLR2 gene mRNAs (P <0.01, P =0.006, P =0.04, respectively), and T-NOS, iNOS and cNOS proteins (P =0.002, P =0.028 and P =0.003, respectively) and increased serum tumor necrosis factor alpha (TNF-α) concentration (P <0.01), but ACTH treatment has no significant effect on them. Neither ACTH nor LPS treatment had any significant effect on levels of pulmonary SOD, CAT and MDA secretion, as well as expression of IL-6, TNF-α, IL-10, COX-2 and TLR4 mRNAs (P >0.05). Pulmonary cortisol level was inhibited by LPS (P =0.034), with a similar trend of inhibition by ACTH (P =0.062). Pretreatment with ACTH tended to downregulate total GR protein levels (P =0.084), and LPS tended to inhibit GR protein expression in the nucleus (P =0.088). Pulmonary expression level of Let-7i targeted TLR4 was significantly increased by ACTH treatment (P =0.013), but not with LPS. These results suggest that LPS may increase the expression of pulmonary TLR2 mRNA by inhibiting nuclear translocation of GC-GR, thus causing a slight inflammatory response in the lungs of pigs, which is not affected by chronic ACTH treatment.

INTRODUCTION

The management and transport of modern farms always induces some persistent stresses in domestic animals. The lipopolysaccharide (LPS) challenge reduced feed consumption and increased plasma pro-inflammatory cytokines of pigs, which was inhibited by high temperature stress (Campos *et al.*, 2014). Long-term effects of social stress have been reported to inhibit antiviral immunity in pigs (de Groot *et al.*, 2001). Generally, exposure to chronic or continuous stress inhibits the host's immune response (Salak-Johnson and McGlone, 2007).

As discussed in our previous work, respiratory mucosa serves as a physicochemical barrier to protect the host against the invasion of airborne pathogens (Sun *et al.*, 2013). The epithelium of the respiratory mucosa can express Toll-like receptors (TLRs) that recognize pathogens. LPS, as a classic ligand for TLR4, stimulates

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the body to produce inflammatory response (Mukesh *et al.*, 2010; Wang *et al.*, 2017). However, little information is available regarding the effect of chronic stress on LPS-induced inflammatory response in the lungs of pigs.

It is well known that the hypothalamic–pituitary–adrenal (HPA) axis is involved in the modulation of inflammatory response, and the immune-HPA axis interaction is critical for the maintenance of homeostasis. TLRs are responsible for the recognition of a variety of pathogens and induces inflammatory responses, which are normally restricted by the HPA axis with a feedback mechanism (Chinenov and Rogatsky, 2007). The HPA axis is known to be activated by a variety of bacterial, viral and inflammatory insults, and then releases glucocorticoid (GC), which acts through a ligand-dependent transcription factor-glucocorticoid receptor (GR) (Chinenov and Rogatsky, 2007; Sun *et al.*, 2013). Chronic stress can stimulate the HPA axis to release adrenocorticotropine hormone (ACTH), and the circulating ACTH then stimulates the adrenal gland that releases GC to regulate inflammation (Verbrugghe *et al.*, 2012). It remains uncertain whether chronic stress can interfere with LPS-induced inflammation via GR.

Continuous ACTH treatment for animals could simulate chronic stress (Gamallo *et al*., 1988) (Haussmann *et al.*, 2000). The present work aims to investigate the effect of chronic stress on LPS-induced inflammatory response in local lung tissue of pig. Therefore, we used a pig model exposed to LPS injection with or without continuous ACTH pretreatment to describe secretions of inflammatory cytokines, enzymes regulating oxidative stress, cortisol, levels of pulmonary TLR2-4, GR mRNA, and protein expression, including expression levels of GR protein in the nucleus and all miRNAs that target TLR2 and TLR4.

MATERIALS AND METHODS

Animals and experimental design

A 2×2 factorial design was used for injection with LPS or saline as one factor and ACTH treatment or saline as the other factor. Twenty-four 30-day-old Duroc × Landrace × Large White crossbred piglets with average weights of 12 ± 0.5 kg (obtained from our partner farm, Zhenjiang Muyuan Animal Science and Technology Development Co., Ltd., Zhenjiang, China).) were randomly assigned to 4 treatments: control group (CC), LPS (E. Coli serotype, Sigma Aldrich, Dublin, Ireland) injection without ACTH (Sigma Aldrich, Dublin, Ireland) pretreatment group (LPS), ACTH alone treatment group (ACTH), LPS injection with ACTH pretreatment group (ACTH+LPS). Each group consisted of 6 male piglets. All pigs were provided with water and food *ad libitum*. The ACTH pigs and ACTH+LPS pigs were injected intramuscularly with ACTH (2.25 IU/kg body weight) for 7 consecutive injections at 6-h intervals. Pigs in the other two groups were mock-injected with saline in the same manner. The LPS pigs and ACTH+LPS pigs were injected intramuscularly with LPS (15 μg/kg weight) immediately after the seventh ACTH treatment. Pigs in the control and ACTH groups were mock-injected with saline. Then, all pigs were euthanized 6 h post-LPS treatment.

Sample collection

The lobus apicalis of the right lung was removed, and the samples were snap frozen in liquid nitrogen and stored at -70°C. Blood was collected from the precaval vein. Blood samples were centrifuged at 1,500×g for 15 min at 4°C, and the serum samples were stored at -20°C.

Cytokine level in serum

Concentrations of TNF-α and IL-10 in serum were measured in duplicate using a commercial TNF-α enzyme-linked immunosorbent assay (ELISA) kit (PTA00, R&D Systems Inc., Minneapolis, MN) and IL-10 ELISA kit (P1000, R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. The detection limits of TNF-α and IL-10 were 2.8 to 5.0 pg/mL and 1.8 to 5.5 pg/mL, respectively.

Biochemical determination of lung tissues

Lung tissues were homogenized in cold radioimmunoprecipitation assay (RIPA) buffer of (50 mM Tris–HCl pH 7.4, containing 10% glycerol, 1.0% Triton-X 100, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA) with the protease inhibitor cocktail (Roche Applied Science). Then, samples were centrifuged at 1,500×g for 15 min at 4°C, and the supernatant was collected. The enzyme activities of total superoxide dismutase (T-SOD), catalase (CAT), inducible nitric oxide synthase (iNOS), total nitric oxide synthase (TNOS), total superoxide dismutase (SOD), and malondialdehyde (MDA) content in the supernatant of lung tissues was determined using diagnostic kits (A001-1-1, A007-1-1, A014-1-1, A014-1-1, A001-3 and A003-1, respectively, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All experiments about these enzymes were also conducted in Nanjing Jiancheng Bioengineering Institute.

Cortisol and IL-6 level in lung tissues

Concentrations of cortisol and IL-6 in the supernatant of lung tissues were measured in duplicate using a commercial ^{125}I-RIA kit (D10PZB and C12PDB, respectively, Beifang Research Institute of Biotechnology,

Beijing, China) according to the manufacturer's instructions. The detection limits of cortisol and IL-6 were 2 ng/mL and 50 pg/mL, respectively. All samples were measured in the same assay to avoid inter-assay variations. The data was collected in Nanjing General Hospital of Nanjing Military Command.

Table I.- Nucleotide sequences of specific primers used in qPCR.

Name	Primers (from 5' to 3')
Interleukin-1β (IL-1β)	F: CAGGGGACTTGAAGAGAG
	R: GCTGATGTACCAGTTGGG
Interleukin-6 (IL-6)	F: CTACTGCCTTCCCTACCC
	R: ACCTCCTTGCTGTTTTCA
Tumor necrosis factor (TNF-α)	F: CCTCTTCTCCTTCCTCCT
	R: ATTGGCATACCCACTCTG
Interleukin-10 (IL-10)	F: CATCCACTTCCCAACCAG
	R: TCCTCCCCATCACTCTCT
Cyclooxygenase-2 (COX-2)	F: GTGTGAAAGGGAGGAAAGA
	R: AAACTGATGGGTGAAGTGC
T oll-like receptor 4 (TLR4)	F: CAACATCCCCACATCAG
	R: CGCTAGGTTTGTCTCAA

Gene expression level

Briefly, total RNA was extracted from lung tissue using Trizol reagent (15596018, Invitrogen, Carlsbad, CA) according to our previous description (Sun *et al.*, 2013). From each sample 1 μg of total RNA was converted to cDNA using PrimeScript® RT reagent kit with gDNA Eraser (DRR047A, Takara, Dalian, China). The primers used are listed in Table I. Also, QPCR reactions and gene expression levels of mRNA were performed using the SYBR Green QPCR Master Mix (DRR041A, TOYOBO Ltd., Japan) as previously described (Sun *et al.*, 2013). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as a reference gene for normalization.

Protein expression level analysis

As described in our previous work (Sun *et al.*, 2013), nuclear, cytosolic, and total protein were extracted from lung tissues, and the protein expression level was analyzed by using Western blot in the same way. In brief, protein was transferred onto nitrocellulose membranes and incubated overnight at 4°C with a primary antibody GR (sc-1004, Santa Cruz Biotechnology, Santa Cruz, CA) or TLR2 (sc-166900, Santa Cruz Biotechnology, Santa Cruz, CA). Lamin A/C (BS1446, Bioworld, China) or β-actin (KC-SA08, Kangcheng, China) was used as a loading control. The membranes were detected using chemiluminescence.

miRNA expression level

All miRNAs that target TLR2 and TLR4 were predicted by computer-aided algorithms from TargetScan (http://www.targetscan.org/vert_42/), and miRNA expression level was performed according to previous publication (Pan *et al.*, 2013). Briefly, total RNA was extracted from lung tissue using Trizol reagent. The RNA (4 μg) was polyadenylated by poly(A) polymerase (PAP) at 37°C for 1 h in a 20 μl reaction mixture using the Poly(A) Tailing Kit (AM1350, Ambion, USA), and tailing reactions were performed. The tailing reaction solution contained 4 μg of RNA samples (1 μg/μl), 2 μl of 25 mM MnCl2, 4 μl of 5×E-PAP buffer, 0.8 μl of E-PAP, 2 μl of 10 mM ATP, and 7.2 μL of nuclease-free water in a 20 μl final volume. Then, tailing RNAs (2 μg) were converted to cDNA using a gene-specific oligo dT-adapter primer (1μg/μl). QPCR reactions were performed using the SYBR Green QPCR Master Mix with a Mx3000P QPCR system (Agilent Technologies, Stratagene, USA). All special miRNA primers were designed based on mature miRNA sequences of pigs in miRbase (http://www.mirbase.org/). All primers used are listed in Table II, including special miRNA primers (ssc-miR-338, ssc-miR-146b, ssc-let-7i), universal primers, exogenous reference primer and oligo dT-adaptor primers.

Table II.- Nucleotide sequences of specific primers used in the study.

Name	Primers (from 5' to 3')	Application
	ATTGGAGGCTATTAAGATGATT	
ssc-miR-338	TCCAGCATCAGTGATTTTGTTG	
ssc-miR-146b	TGAGAACTGAATTCCATAGGC	
ssc-let-7i	TGAGGTAGTAGTTTGTGCT	miRNA expression
Oligo dT-adaptor	TAGAGTGAGTGTAGCGAGCACAGAATTAATA CGACTCACTATAGGTTTTTTTTTTTTTTTTVN	
exogenous reference	GTGACCCACGATGTGTATTCGC	
universal	TAGAGTGAGTGTAGCGAGCA	

Statistical analysis

Descriptive statistics were performed to check the normality and homogeneity of variances before using parametric analyses. All data were analyzed by two-way analysis of variance (ANOVA) in the general linear model (GLM) procedure of SPSS 16.0 (SPSS Inc., Chicago, IL, USA) with LPS and ACTH treatments as main effects. For interaction between factors, a one-way ANOVA analysis was performed. Duncan's test was used as the post hoc test. Two-tailed *P*-values with $P \leq 0.05$ were considered significant. Data are expressed as mean ± SEM.

RESULTS

Serum cytokine level

Treatment with LPS increased levels of TNF-α in serum ($P < 0.01$), but ACTH injection has no significant effect on them. No significant interaction was observed between the two treatments ($P > 0.05$) (Fig. 1). Secretion of IL-10 protein was not detected in serum.

Inflammation and cortisol level in lung

As shown in Table III, LPS treatment activated expressions of IL-1β and IL-10 gene mRNA ($P < 0.01$ and $P = 0.006$, respectively), and ACTH treatment had no significant effect on them. There was no significant interaction between ACTH and LPS treatments for expressions of the two genes. As for other genes related to inflammation, no significant differences were observed in the expression of IL-6, TNF-α, IL-10, and COX-2 mRNAs post ACTH or LPS treatment ($P > 0.05$).

Pulmonary cortisol level was inhibited by LPS ($P = 0.034$), with a similar trend of inhibition by ACTH ($P = 0.062$). However, there was no significant interaction between ACTH and LPS treatments ($P > 0.05$).

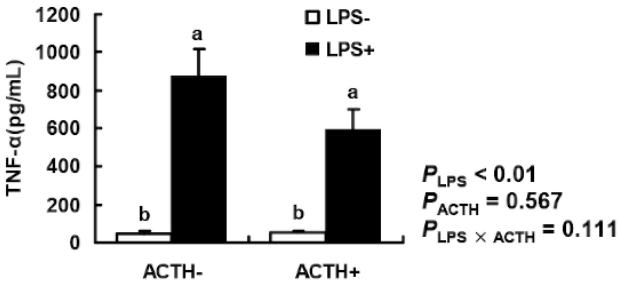

Fig. 1. Effects of LPS/ACTH on serum TNF-α concentration. Pigs were administered LPS injection with or without continuous ACTH pretreatment. TNF-α protein level in serum was determine by ELISA. TNF-α, Tumor necrosis factor; LPS, lipopolysaccharide; ACTH, adrenocorticotropine. Mean values with different letters (a, b, c, or d) are significantly different ($P < 0.05$) from each other. n = 5-6.

Oxidative stress in lung

As shown in Table IV, LPS treatment increased levels of T-NOS, iNOS and cNOS protein secretion ($P = 0.002$, $P = 0.028$ and $P = 0.003$, respectively), but ACTH treatment had no significant effect on them. There was a significant interaction between ACTH and LPS treatments for T-NOS and cNOS ($P = 0.038$ and $P = 0.01$, respectively). Levels of pulmonary SOD, CAT and MDA secretion were not affected by ACTH or LPS treatment ($P > 0.05$).

Table III.- Effects of LPS/ACTH on inflammation and cortisol levels in lung tissue.

Items[2]		Treatments[1]				SEM[3]	P–values[4]		
		ACTH -		ACTH +					
		LPS-	LPS+	LPS-	LPS+		ACTH	LPS	ACTH×LPS
Genes mRNA expression	IL-1β	1.00[b]	2.52[a]	0.79[b]	3.55[a]	0.22	0.055	< 0.01	0.052
	IL-6	1.00	1.01	0.93	1.18	0.07	0.693	0.309	0.337
	TNF-α	1.00	0.95	0.72	0.98	0.09	0.443	0.538	0.347
	IL-10	1.00	2.42	0.82	2.24	0.31	0.447	0.006	0.75
	COX-2	1.00	0.82	0.64	0.92	0.07	0.624	0.358	0.2
IL-6 (pg/mg protein)		231.64	176.46	181.04	184.44	12.77	0.415	0.324	0.266
Cortisol(ng/mg protein)		23.63	15.89	16.46	15.36	0.97	0.062	0.034	0.103

[1] Pigs were administered LPS injection with or without continuous ACTH pretreatment. n = 5-6.
[2] IL-1β, Interleukin-1β; IL-6, Interleukin-6; TNF-α, Tumor necrosis factor; IL-10, Interleukin-10; COX-2, cyclooxygenase-2.
[3] SEM, standard error of mean.
[4] Mean values with same superscripts in the same line or row differ insignificantly ($P > 0.05$), while those with different superscripts in succession differ significantly ($P < 0.05$) and those with alternative superscripts differ very significantly ($P < 0.01$).

Table IV.- Effects of LPS/ACTH on oxidative stress in lung tissue.

Items[2]	Treatments[1]				SEM[3]	*P*–values[4]		
	ACTH -		ACTH +					
	LPS-	LPS+	LPS-	LPS+		ACTH	LPS	ACTH×LPS
T-NOS (U/mg protein)	0.57^{bc}	0.64^{ab}	0.47^{c}	0.77^{a}	0.03	0.86	0.002	0.038
iNOS (U/mg protein)	0.14	0.19	0.17	0.19	0.01	0.255	0.028	0.412
cNOS (U/mg protein)	0.44^{b}	0.46^{ab}	0.30^{c}	0.58^{a}	0.02	0.864	0.003	0.01
MDA(nmoL/mg protein)	0.79	1.00	0.86	0.89	0.04	0.788	0.145	0.283
SOD (U/mg protein)	19.89	19.88	16.92	21.00	0.84	0.587	0.239	0.238
CAT (U/mg protein)	1.77	1.72	1.72	1.77	0.08	0.999	0.999	0.763

[1] Pigs were administered LPS injection with or without continuous ACTH pretreatment. n = 5-6.
[2] T-NOS, total nitric oxide synthase; i-NOS, inducible nitric oxide synthase; c-NOS, primary nitric oxide synthase; MDA, malondialdehyde; SOD, total superoxide dismutase; CAT, catalase.
[3] SEM, standard error of mean.
[4] Mean values with same superscripts in the same line or row differ insignificantly (P >0.05), while those with different superscripts in succession differ significantly (P <0.05) and those with alternative superscripts differ very significantly (P <0.01).

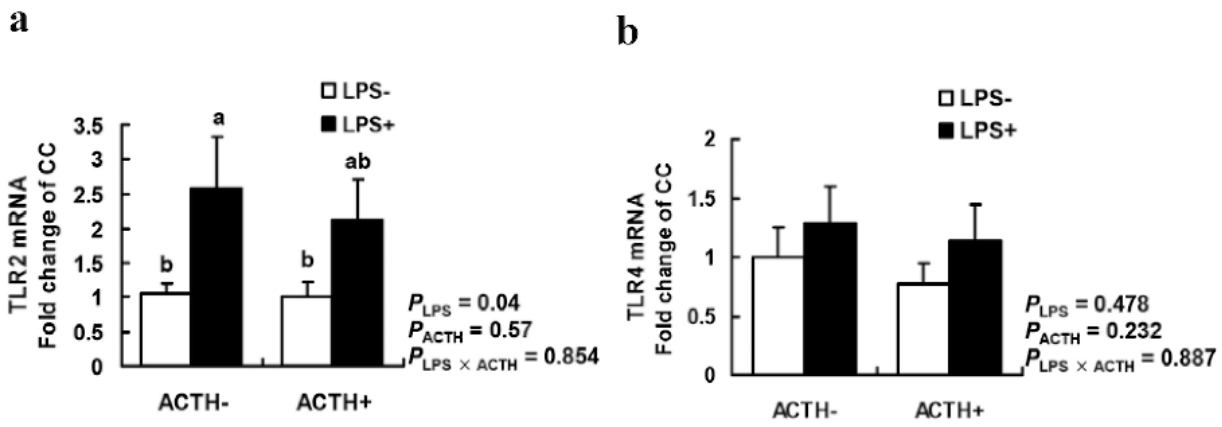

Fig. 2. Expression of TLR2 mRNA (a) TLR4 mRNA (b) in porcine lung tissue. Pigs were administered LPS injection with or without continuous ACTH pretreatment. TNF-α protein level in serum was determine by ELISA. TLR, toll-like receptor 2; LPS, lipopolysaccharide; ACTH, adrenocorticotropine hormone. b-actin was chosen as a reference for normalization in the qPCR and Western blot analyses. Mean values with different letters (a, b, c, or d) are significantly different (P <0.05) from each other. n = 5-6.

Levels of pulmonary TLR2, 4 mRNA expression

Treatment with LPS increased the level of pulmonary TLR2 mRNA expression (P =0.04), but ACTH had no significant effect on them. There was no significant interaction between the two treatments (P >0.05) (Fig. 2a). Neither ACTH nor LPS had any significant effect on TLR4 mRNA expression (Fig. 2b). Secretion of IL-10 protein was not detected in serum.

Levels of pulmonary GR mRNA and protein expression

As shown in Figure 3b, ACTH pretreatment tended to downregulate the expression of total GR protein (P =0.084), but LPS treatment had no significant effect on it. A downward tendency was observed in nuclear GR protein expression post-LPS pretreatment (P =0.088), but no significant effect was found post ACTH treatment (Fig. 3d). Neither ACTH nor LPS treatment had a significant effect on pulmonary GR mRNA or cytoplasmic GR protein expression (Fig. 3a and c).

Expressions of miRNA

All miRNAs that target TLR2 and TLR4 were predicted. The result showed that miR-338, miR-146a, miR-146b, and Let-7i had potential regulatory effects on TLR4. As shown in Table V, pulmonary expression level of Let-7i was significantly increased by treatment with ACTH (P = 0.013), but not with LPS. However, neither ACTH nor LPS treatment had any significant effect on the expression of miR-338, miR-146a and miR-146b. No potential miRNA was found for TLR2.

Table V.- The expression of miRNAs targeting TLR4 genes in the lung tissue.

miRNA	Treatments[1]				SEM[2]	P–values[3]		
	ACTH -		ACTH +					
	LPS -	LPS+	LPS-	LPS+		ACTH	LPS	ACTH×LPS
ssc-miR-338	1.00	0.66	1.34	1.67	0.16	0.073	0.862	0.263
ssc-miR-146a	1.00	1.35	0.80	0.65	0.31	0.476	0.897	0.706
ssc-miR-146b	1.00	2.07	0.75	0.98	0.31	0.246	0.337	0.595
Let-7i	1.00	0.70	1.64	1.83	0.16	0.013	0.870	0.438

[1] Pigs were administered LPS injection with or without continuous ACTH pretreatment. n = 5-6.
[2] SEM, standard error of mean.
[3] Mean values with different letters (a, b, c, or d) are significantly different (P <0.05) from each other. n = 5-6.

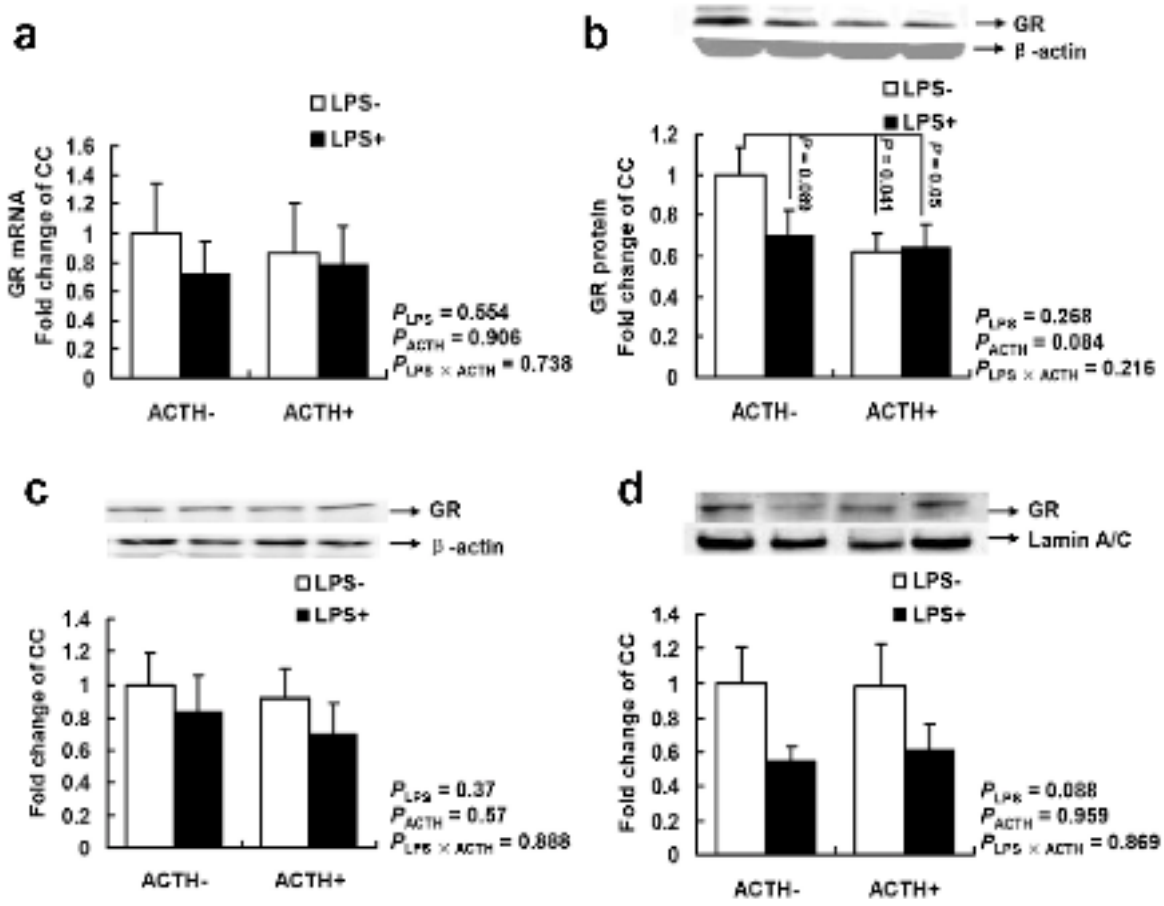

Fig. 3. GR mRNA (a) total GR protein (b) cytoplasmic (c) and nuclear (d) GR protein content in porcine lung tissue.

DISCUSSION

LPS is known to promote the release of cytokines and stimulate inflammatory response. For example, it has been reported that levels of pulmonary pro-inflammatory cytokines, such as TNF-α, IL-1β, and granulocyte-macrophage colony-stimulating factor, were elevated in mice treated with aerosol LPS (Matteri *et al.*, 1998). In a previous study, concentrations of TNF-α, IL-1β, and IL-6 in mammary tissues were increased after an intramammary infusion of LPS (Miao *et al.*, 2007). Correspondingly, in our pro-inflammatory model, the level of TNF-α in serum was increased after intramuscular injection of LPS, which

indicated that the whole-body system of pigs was sensitive to LPS treatment. However, in all the cytokines detected in our experiment, LPS only increased concentrations of IL-1β and IL-10 mRNA in lung tissues, while the expression levels of other cytokines remained unchanged. This suggests that there was only a slight inflammatory response in the lungs at this time, which is not consistent with previous reports. This may be due to the different treatment methods of LPS. In previous studies, the lungs or breast tissues were directly treated with LPS; therefore, a strong inflammatory response was caused. However, in our study, the method of LPS treatment was intramuscular injection, which only caused a mild inflammatory response in local lung tissues. It was also reported that the cytokine response induced by LPS was age- and tissue-dependent in neuroendocrine tissues of newborn pigs (Matteri *et al.*, 1998). Therefore, the specificity of age and tissue may have impacted the inflammatory response of pig lungs in our experiment.

The inflammatory response was stimulated by LPS mainly through activation of TLR4. Then, TLR4 activates signal transduction pathways, such as nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK), through a series of signal cascade reactions, and ultimately activates the inflammatory response (Barton and Medzhitov, 2003; Nasu and Narahara, 2010). A large number of studies have used LPS to induce pro-inflammatory reactions when studying the mechanisms relative to TLR4 (Juarranz *et al.*, 2006; Kim *et al.*, 2012; Wang *et al.*, 2009; Willis *et al.*, 2010). However, in our experiment, LPS treatment had no significant effect on the expression of TLR4 mRNA but increased the expression of TLR2 mRNA. In fact, LPS can activate the TLR2 pathway. It has been found that knockout of TLR2 can inhibit the pro-inflammatory response of LPS, and decrease the secretion of cytokines, such as IL-1, TNF-α, IL-6, and other cytokines in the adrenal gland and serum (Bornstein *et al.*, 2004). It was also found that TLR2 mRNA expression was upregulated in LPS-induced hemorrhagic shock of mice (Fan *et al.*, 2007). It was reported that TLR4 was approximately 100-fold more sensitive to LPS than TLR2 in human embryonic kidney 293 cells, which depended on CD14 and LPS-binding protein (Muta and Takeshige, 2001). A higher level of TLR4 expression was associated with higher sensitivity to LPS, which has also been demonstrated by overexpression of TLR4 in transgenic mice (Levy *et al.*, 2009; Bihl *et al.*, 2003). Therefore, in our study, only TLR2 mRNA was upregulated, which was consistent with a weaker inflammatory reaction. This suggests that the inflammatory reaction in the lung may only be activated by the TLR2 pathway.

Many studies have demonstrated that TLRs were activated in organ injury, which was associated with oxidative stress (Pushpakumar *et al.*, 2017; Lee and Kang, 2017). Various inflammatory cytokines can induce the release of reactive oxygen species and formation of toxic metabolites, such as MDA (Zhu *et al.*, 2017). NO, as an important inflammatory mediator, is produced by iNOS (Li *et al.*, 2017). Here, we found that LPS treatment increased levels of pulmonary T-NOS, iNOS, and cNOS, but had no significant effect on levels of SOD, CAT, and MDA. These results suggest that LPS may only promote the production of NO in local lung tissue but does not induce lipid peroxidation. Meanwhile, ACTH treatment has no significant effect on levels of TLR2, TLR4, cytokine, iNOS, and MDA. All these results suggest that the chronic stress simulated by continuous ACTH intramuscular injection has no significant effect on oxidative stress and inflammatory response in the local lung tissue of pigs. However, we also found that ACTH pretreatment down-regulated LPS-induced expressions of TNF-α, COX-2, TLR2 and TLR4 mRNA, as well as IL-6 protein level in porcine adrenal gland (data not shown). Therefore, we speculated that effect of ACTH on LPS-induced inflammation is tissue-specific.

In the cytoplasm, unliganded GR in the inactivation state is combined with heat shock protein 90 (Pratt *et al.*, 2004). GC is a small hydrophobic molecule, which can easily pass through the cell membrane and combine with GR in the cytoplasm, forming GC-GR complex in the nucleus (Pratt *et al.*, 2004; Bledsoe *et al.*, 2004). The GC-GR complex finally transcribes and regulates the expression of innate immune molecules in the nucleus and plays an anti-inflammatory and anti-immune role. We found that pulmonary cortisol levels and nuclear GR protein expression were inhibited by LPS. This result is consistent with significant upregulation of lung TLR2 mRNA and mild inflammatory response in the lungs. This suggests that LPS may increase the expression of TLR2 by inhibiting the anti-inflammatory effects of GC-GR, thus causing a slight inflammatory response in the lungs of pigs. Here, we also found that ACTH pretreatment had a tendency to downregulate the expression of pulmonary cortisol and total GR protein but had no effect on nuclear GR protein expression. This suggests that GC-GR translocation into the nucleus may not be affected by chronic ACTH treatment, so that inflammatory response in the lungs is not changed.

Many reports on the effects of ACTH treatment on cortisol were not consistent. For example, high doses of ACTH in infantile spasms caused a rapid increase of serum cortisol (Snead *et al.*, 1989). Serum cortisol levels were slightly increased in healthy adults from the third to the fifth day during chronic ACTH treatment,

but decreased in patients with multiple sclerosis (Maida and Summer, 1979). Plasma steroid levels of guinea pigs receiving chronic ACTH treatment were activated with a brief upregulation after the last injection of ACTH and then returned to the basic level (Provencher *et al.*, 1992). Thus, the effect of ACTH on cortisol release is associated with the species and type of stress. Here, we found that ACTH pretreatment had a tendency to downregulate the expression of pulmonary cortisol levels. Cortisol in the body may be metabolized locally in the tissue by 11β-hydroxysteroid-dehydrogenases 1 and 11β-hydroxysteroid-dehydrogenases 2, which activates and inactivates GCs, respectively (Holmes *et al.*, 2003). Therefore, we speculate that the activity of both enzymes in the lungs was altered by chronic ACTH treatment, resulting in a decrease of pulmonary cortisol levels. Moreover, as we discussed in a previous report, GC/GR interaction is highly dynamic (Sun *et al.*, 2013), and release of GC is rhythmic and pulsating with a peak approximately each hour (Biddie *et al.*, 2012). Therefore, single time point sampling, as used in the present study, may produce highly individual variations due to unsynchronized GC pulsatility.

The miRNAs, as a class of endogenous non-coding RNA, can regulate inflammation through inhibiting mRNA transcription or promoting mRNA degradation of target gene. In present study, LPS treatment had no significant effect on expression of all the miRNA predicted. Thus, we speculate that pulmonary inflammation was not mediated by miRNAs post LPS treatment. Previous studies have shown Let-7i, as a member of the let-7 miRNA family, can decrease TLR4 expression and inhibit inflammation and oxidative stress (Qi *et al.*, 2016; Xiang *et al.*, 2017). Here, ACTH significantly increased pulmonary expression level of Let-7i. But inconsistent with expectation, TLR4 mRNA levels were not affected. Actually, a single miRNA can regulate thousands of target genes to control innate and adaptive immune response (Singh *et al.*, 2013). This suggests that ACTH-induced increase of Let-7i expression level may target other gene to modulate immune activity in lung of pig.

GR can take part in modulating mRNA expressions of a series of inflammatory gene via complex mechanisms of transcriptional regulation. It can directly or indirectly regulate target genes by itself or interfering with other transcriptional factors (Beck *et al.*, 2009; Biddie *et al.*, 2012). Our previous experiments have proved that vaccination of inactivated vaccine resulted in diminished TLR2 expression via the inhibition of GR nuclear translocation and binding to the TLR2 promoter (Sun *et al.*, 2013). It has also been showed that GR interfered with the transcriptional activity of NF-κB and Activator protein 1 (AP-1), thus inhibiting the expression of inflammatory molecules such as IL-2, IL-6, IL-8, TNF-α and iNOS (De Bosscher *et al.*, 2003). Therefore, further in vitro investigations are needed to delineate the complex regulatory network of GR and inflammatory molecules post nuclear translocation of GR.

CONCLUSION

Taken together, we first in vivo determined that LPS may increase the expression of pulmonary TLR2 mRNA by inhibiting nuclear translocation of GC-GR, thus causing a slight inflammatory response in the lungs of pigs. ACTH pretreatment-stimulated stress tended to down-regulate pulmonary cortisol and total GR protein levels, but had no effect on LPS-induced slight inflammation in local pig lungs. Our study will deepen the knowledge about the relationship between immune response and stress during inflammation, and may provide a theoretical basis for using this relationship as a useful alert for porcine health and welfare.

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Declarations of interest

The authors declare no conflicts of interest.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University. The protocol of this study was reviewed and approved specifically, with the project number 2012CB124704. The slaughter and sampling procedures strictly followed the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set

by the Ministry of Science and Technology, China and the "Regulation regarding the Management and Treatment of Experimental Animals" (2008) No. 45 set by the Jiangsu Provincial People's Government.

REFERENCES

Barton, G.M. and Medzhitov, R., 2003. Toll-like receptor signaling pathways. *Science*, **300**: 1524-1525. https://doi.org/10.1126/science.1085536

Beck, I.M., Vanden Berghe, W., Vermeulen, L., Yamamoto, K.R., Haegeman, G. and De Bosscher, K., 2009. Crosstalk in inflammation: The interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. *Endocr. Rev.*, **30**: 830-882. https://doi.org/10.1210/er.2009-0013

Biddie, C., Conway-Campbell, Becky L. and Stafford L., 2012. Dynamic regulation of glucocorticoid signalling in health and disease. *Rheumatology*, **51**: 403-412. https://doi.org/10.1093/rheumatology/ker215

Bihl, F., Salez, L., Beaubier, M., Torres, D., Lariviere, L., Laroche, L., Benedetto, A., Martel, D., Lapointe, J.M., Ryffel, B. and Malo, D., 2003. Overexpression of toll-like receptor 4 amplifies the host response to lipopolysaccharide and provides a survival advantage in transgenic mice. *J. Immunol.*, **170**: 6141-6150. https://doi.org/10.4049/jimmunol.170.12.6141

Bledsoe, R.K., Stewart, E.L. and Pearce, K.H., 2004. Structure and function of the glucocorticoid receptor ligand binding domain. *Vitam. Horm.*, **68**: 49-91. https://doi.org/10.1016/S0083-6729(04)68002-2

Bornstein, S.R., Zacharowski, P., Schumann, R.R., Barthel, A., Tran, N., Papewalis, C., Rettori, V., Mccann, S.M., Schulze-Osthoff, K., Scherbaum, W.A., Tarnow, J. and Zacharowski, K., 2004. Impaired adrenal stress response in Toll-like receptor 2-deficient mice. *Proc. natl. Acad. Sci. USA*, **101**: 16695-16700. https://doi.org/10.1073/pnas.0407550101

Campos, P.H., Merlot, E., Damon, M., Noblet, J. and Le Floc'h, N., 2014. High ambient temperature alleviates the inflammatory response and growth depression in pigs challenged with *Escherichia coli* lipopolysaccharide. *Vet. J.*, **200**: 404-409. https://doi.org/10.1016/j.tvjl.2014.04.001

Chinenov, Y. and Rogatsky, I., 2007. Glucocorticoids and the innate immune system: crosstalk with the toll-like receptor signaling network. *Mol. Cell Endocrinol.*, **275**: 30-42. https://doi.org/10.1016/j.mce.2007.04.014

De Bosscher, K., Vanden Berghe, W. and Haegeman, G., 2003. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr. Rev.*, **24**: 488-522. https://doi.org/10.1210/er.2002-0006

De Groot, J., Ruis, M.A., Scholten, J.W., Koolhaas, J.M. and Boersma, W.J., 2001. Long-term effects of social stress on antiviral immunity in pigs. *Physiol. Behav.*, **73**: 145-158. https://doi.org/10.1016/S0031-9384(01)00472-3

Fan, J., Li, Y., Vodovotz, Y., Billiar, T.R. and Wilson, M.A., 2007. Neutrophil NAD(P)H oxidase is required for hemorrhagic shock-enhanced TLR2 up-regulation in alveolar macrophages in response to LPS. *Shock*, **28**: 213-218. https://doi.org/10.1097/shk.0b013e318033ec9d

Gamallo, A., Alario, P., Villanua, M.A. and Nava, M.P., 1988. Effect of chronic stress in the blood pressure in the rat: ACTH administration. *Horm. Metab. Res.*, **20**: 336-338. https://doi.org/10.1055/s-2007-1010830

Haussmann, M.F., Carroll, J.A., Weesner, G.D., Daniels, M.J., Matteri, R L. and Lay, D.C., 2000. Administration of ACTH to restrained, pregnant sows alters their pigs' hypothalamic-pituitary-adrenal (HPA) axis. *J. Anim. Sci.*, **78**: 2399-2411. https://doi.org/10.2527/2000.7892399x

Holmes, M.C., Yau, J.L., Kotelevtsev, Y., Mullins, J.J. and Seckl, J.R., 2003. 11 Beta-hydroxysteroid dehydrogenases in the brain: two enzymes two roles. *Annls. N.Y. Acad. Sci.*, **1007**: 357-366. https://doi.org/10.1196/annals.1286.035

Juarranz, Y., Gutierrez-Canas, I., Arranz, A., Martinez, C., Abad, C., Leceta, J., Pablos, J.L. and Gomariz, R.P., 2006. VIP decreases TLR4 expression induced by LPS and TNF-alpha treatment in human synovial fibroblasts. *Annls. N.Y. Acad. Sci.*, **1070**: 359-364. https://doi.org/10.1196/annals.1317.045

Kim, I.T., Ryu, S., Shin, J.S., Choi, J.H., Park, H.J. and Lee, K.T., 2012. Euscaphic acid isolated from roots of Rosa rugosa inhibits LPS-induced inflammatory responses via TLR4-mediated NF-kappaB inactivation in RAW 264.7 macrophages. *J. Cell Biochem.*, **113**: 1936-1946. https://doi.org/10.1002/jcb.24062

Lee, S.I. and Kang, K.S., 2017. Function of capric acid in cyclophosphamide-induced intestinal inflammation, oxidative stress, and barrier function in pigs. *Sci. Rep.*, **7**: 16530. https://doi.org/10.1038/s41598-017-16561-5

Levy, E., Xanthou, G., Petrakou, E., Zacharioudaki,

V., Tsatsanis, C., Fotopoulos, S. and Xanthou, M., 2009. Distinct roles of TLR4 and CD14 in LPS-induced inflammatory responses of neonates. *Pediatr. Res.*, **66**: 179-184. https://doi.org/10.1203/PDR.0b013e3181a9f41b

Li, H., Yoon, J.H., Won, H.J., Ji, H.S., Yuk, H.J., Park, K.H., Park, H.Y. and Jeong, T.S., 2017. Isotrifoliol inhibits pro-inflammatory mediators by suppression of TLR/NF-kappaB and TLR/MAPK signaling in LPS-induced RAW264.7 cells. *Int. Immunopharmacol.*, **45**: 110-119. https://doi.org/10.1016/j.intimp.2017.01.033

Maida, E. and Summer, K., 1979. Serum cortisol levels of multiple sclerosis patients during ACTH treatment. *J. Neurol.*, **220**: 143-148. https://doi.org/10.1007/BF00313955

Matteri, R.L., Klir, JJ., Fink, B.N. and Johnson, R.W., 1998. Neuroendocrine-immune interactions in the neonate. *Domest. Anim. Endocrinol.*, **15**: 397-407. https://doi.org/10.1016/S0739-7240(98)00024-1

Miao, J.F., Zhu, Y.M., Gu, B.B., Wang, X.B., Zou, S.X. and Deng, Y.E., 2007. Evaluation of the changes of immune cells during lipopolysaccharide-induced mastitis in rats. *Cytokine*, **40**: 135-143. https://doi.org/10.1016/j.cyto.2007.08.012

Mukesh, M., Bionaz, M., Graugnard, D.E., Drackley, J.K. and Loor, J.J., 2010. Adipose tissue depots of Holstein cows are immune responsive: inflammatory gene expression *in vitro*. *Domest. Anim. Endocrin.*, **38**: 168-178. https://doi.org/10.1016/j.domaniend.2009.10.001

Muta, T. and Takeshige, K., 2001. Essential roles of CD14 and lipopolysaccharide-binding protein for activation of toll-like receptor (TLR)2 as well as TLR4 Reconstitution of TLR2- and TLR4-activation by distinguishable ligands in LPS preparations. *Eur. J. Biochem.*, **268**: 4580-4589. https://doi.org/10.1046/j.1432-1327.2001.02385.x

Nasu, K. and Narahara, H., 2010. Pattern recognition via the toll-like receptor system in the human female genital tract. *Mediat. Inflamm.*, **2010**: 976024. https://doi.org/10.1155/2010/976024

Pan, S., Zheng, Y., Zhao, R. and Yang, X., 2013. miRNA-374 regulates dexamethasone-induced differentiation of primary cultures of porcine adipocytes. *Horm. Metab. Res.*, **45**: 518-525. https://doi.org/10.1055/s-0033-1334896

Pratt, W.B., Galigniana, M.D., Morishima, Y. and Murphy, P.J., 2004. Role of molecular chaperones in steroid receptor action. *Essays Biochem.*, **40**: 41-58. https://doi.org/10.1042/bse0400041

Provencher, P.H., Tremblay, Y., Caron, S. and Belanger, A., 1992. Effect of chronic ACTH treatment on guinea-pig adrenal steroidogenesis: steroid plasma levels, steroid adrenal levels, activity of steroidogenic enzymes and their steady-state mRNA levels. *J. Steroid Biochem. Mol. Biol.*, **41**: 69-78. https://doi.org/10.1016/0960-0760(92)90226-9

Pushpakumar, S., Ren, L., Kundu, S., Gamon, A., Tyagi, S.C. and Sen, U., 2017. Toll-like receptor 4 deficiency reduces oxidative stress and macrophage mediated inflammation in hypertensive kidney. *Sci. Rep.*, **7**: 6349. https://doi.org/10.1038/s41598-017-06484-6

Qi, M., Yin, L., Xu, L., Tao, X., Qi, Y., Han, X., Wang, C., Xu, Y., Sun, H., Liu, K. and Peng, J., 2016. Dioscin alleviates lipopolysaccharide-induced inflammatory kidney injury via the microRNA let-7i/TLR4/MyD88 signaling pathway. *Pharmacol. Res.*, **111**: 509-522. https://doi.org/10.1016/j.phrs.2016.07.016

Salak-Johnson, J. and Mcglone, J., 2007. Making sense of apparently conflicting data: stress and immunity in swine and cattle. *J. Anim. Sci.*, **85**: E81-E88. https://doi.org/10.2527/jas.2006-538

Singh, R.P., Massachi, I., Manickavel, S., Singh, S., Rao, N.P., Hasan, S., Mc Curdy, D.K., Sharma, S., Wong, D., Hahn, B.H. and Rehimi, H., 2013. The role of miRNA in inflammation and autoimmunity. *Autoimmu. Rev.*, **12**: 1160-1165. https://doi.org/10.1016/j.autrev.2013.07.003

Snead, O.C., Benton, J.W., Hosey, L.C., Swann, J.W., Spink, D., Martin, D. and Rej, R., 1989. Treatment of infantile spasms with high-dose ACTH efficacy and plasma levels of ACTH and cortisol. *Neurology*, **39**: 1027-1027. https://doi.org/10.1212/WNL.39.8.1027

Sun, Z., Liu, M., Zou, H., Li, X., Shao, G. and Zhao, R., 2013. Vaccination inhibits TLR2 transcription via suppression of GR nuclear translocation and binding to TLR2 promoter in porcine lung infected with *Mycoplasma hyopneumoniae*. *Vet. Microbiol.*, **167**: 425-433. https://doi.org/10.1016/j.vetmic.2013.08.022

Verbrugghe, E., Boyen, F., Gaastra, W., Bekhuis, L., Leyman, B., Van Parys, A., Haesebrouck, F. and Pasmans, F., 2012. The complex interplay between stress and bacterial infections in animals. *Vet. Microbiol.*, **155**: 115-127. https://doi.org/10.1016/j.vetmic.2011.09.012

Wang, P., Han, X., Mo, B., Huang, G. and Wang, C., 2017. LPS enhances TLR4 expression and IFNgamma production via the TLR4/IRAK/NFkappaB signaling pathway in rat pulmonary

arterial smooth muscle cells. *Mol. Med. Rep.*, **16**: 3111-3116. https://doi.org/10.3892/mmr.2017.6983

Wang, W., Xu, M., Zhang, Y.Y. and He, B., 2009. Fenoterol, a beta(2)-adrenoceptor agonist, inhibits LPS-induced membrane-bound CD14, TLR4/CD14 complex, and inflammatory cytokines production through beta-arrestin-2 in THP-1 cell line. *Acta Pharmacol. Sin.*, **30**: 1522-1528. https://doi.org/10.1038/aps.2009.153

Willis, L.M., Bielinski, D.F., Fisher, D.R., Matthan, N.R. and Joseph, J.A., 2010. Walnut extract inhibits LPS-induced activation of BV-2 microglia via internalization of TLR4: Possible involvement of phospholipase D2. *Inflammation*, **33**: 325-333. https://doi.org/10.1007/s10753-010-9189-0

Xiang, W., Tian, C., Peng, S., Zhou, L., Pan, S. and Deng, Z., 2017. Let-7i attenuates human brain microvascular endothelial cell damage in oxygen glucose deprivation model by decreasing toll-like receptor 4 expression. *Biochem. biophs. Res. Commun.*, **493**: 788-793. https://doi.org/10.1016/j.bbrc.2017.08.093

Zhu, N., Cai, C., Zhou, A., Zhao, X., Xiang, Y. and Zeng, C., 2017. Schisandrin B prevents hind limb from ischemia-reperfusion-induced oxidative stress and inflammation via MAPK/NF-kappaB pathways in rats. *BioMed. Res. Int.*, **2017**: 4237973 https://doi.org/10.1155/2017/4237973