The Veterinary Journal 187 (2011) 229–233

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Transportation stress alters the expression of immunoregulatory cytokines in the porcine thymus

Qiongxia Lv [a], Shuxia Zhang [a,*], Ruqian Zhao [b]

[a] The Laboratory of Animal Pathology, Department of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China
[b] The Key Laboratory of Animal Physiology and Biochemistry, Department of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China

ARTICLE INFO

Article history:
Accepted 6 December 2009

Keywords:
Pigs
Thymus
Serum
Cytokines
Transportation stress

ABSTRACT

This study investigated the effects of transportation stress on blood concentrations of the main pro-inflammatory cytokines (interleukins IL-1β, IL-2 and IL-6; tumour necrosis factor-α) and anti-inflammatory cytokines (IL-4 and IL-10) and the expression of these cytokines and their receptors in the thymus. Pigs were assessed after 1, 2 and 4 h of transportation ($n = 5$ per group), with normal housing conditions as a control ($n = 4$). Serum concentrations of IL-2, IL-6 and IL-10 were highest at 1 h, whereas concentrations of IL-6 and IL-10 were significantly decreased at 4 h. Expression of these three cytokines and their receptors was also significantly altered in the thymus during transportation stress. Serum IL-10 concentrations and thymus IL-10 mRNA expression were significantly correlated. The thymus may contribute towards the regulation of cytokines in pigs during transportation.

Introduction

Evidence from animal models and human studies supports the concept that acute stress can have immunomodulatory effects (Padgett and Glaser, 2003). These effects vary among individuals and are dependent upon the nature and duration of the stress and the immune parameter being assessed (Paik et al., 2000; Dhabhar, 2003; Padgett and Glaser, 2003). Transportation is considered to be a major stressor for pigs (Knowles and Warriss, 2000; von Borell, 2001), resulting in deleterious effects on the health, well-being and performance of transported animals. This stressor may also influence the quality of the final animal product (Geverink et al., 1998). However, the response and tolerance of the immune system of pigs to transportation are poorly understood.

Cytokines are important for regulating immune and inflammatory responses (Yang et al., 2008). Immunological challenge with a stressor results in the release of the pro-inflammatory cytokines tumour necrosis factor (TNF) α, interleukin (IL)-1 and IL-6, as well as anti-inflammatory cytokines, from tissues (Appels et al., 2000; Meagher et al., 2007).

The thymus is an important immune organ that produces cytokines, either spontaneously or following stimulation (Yarilin and Belyakov, 2004). Although the role of cytokines in the regulation of inflammation and immunity is well established, data on the effects of transportation stress on thymic cytokine expression and the relationship between thymic production and peripheral serum cytokine concentrations are limited. In the present study, we investigated the impact of acute transportation stress on serum concentrations and thymic expression of pro-inflammatory and anti-inflammatory cytokines in pigs.

Materials and methods

Animals and experimental design

Nineteen male pigs with body weights of 50 ± 2 kg (mean ± standard deviation; *SD*) were randomly divided into three groups of five pigs each and one control group of four pigs. Each group was housed in a separate pen (4.5 m × 2.5 m) at 20 ± 2 °C with artificial light from 07:00 to 21:00 each day. Water and food were available ad libitum.

At 07:00 on the day of transportation, pigs were placed in individual cages and loaded onto a truck. The transportation trial consisted of a continuous 1, 2 or 4 h journey on a country road at a speed of 30–40 km/h. The control group was kept under normal housing conditions. After transportation, all animals were anaesthetised with 10 mg/kg 3% pentobarbital sodium by jugular injection while in the truck or in the housing unit (controls). According to a strict protocol, they were brought to the dissection room, where blood samples were collected by exsanguination. Serum was frozen at −20 °C until analysed. The thymus was removed and frozen in liquid nitrogen. All samples were collected within 20 min (anaesthesia 5–6 min, slaughtering and blood samples 6–8 min, collection of samples of thymus 5–6 min) after transportation. The study protocol was approved by the institutional Animal Care and Use Committee (SCXK, Shanghai, 2005–0002).

Determination of serum cytokine concentrations

Serum concentrations of IL-1β, IL-2, IL-4, IL-6, IL-10 and TNF-α were measured using homologous specific ELISA kits (Adlitteram Diagnostic Laboratories; limit of detection 0.1 ng) according to the manufacturer's instructions. Individual serum samples were thawed in a refrigerator at 4 °C. Specific antibodies were coated in individual wells, standards and samples were dispensed into the appropriate wells,

* Corresponding author. Tel.: +86 2584395316; fax: +86 2584398669.
E-mail address: zsxnjau@yahoo.com.cn (S. Zhang).

1090-0233/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2009.12.004

the enzyme-conjugated reagent was added and plates were mixed gently for 15 s prior to incubation at 36 ± 2 °C for 60 min. The incubation mixture was then removed, wells were rinsed five times with deionised water and residual water droplets were removed by striking the plate sharply onto absorbent paper. A 3,3′,5,5′ tetramethyl-benzidine substrate solution (200 mg/L) was added to the wells, mixed gently for 5 s and then plates were incubated at 36 ± 2 °C for 15 min. The reaction was stopped by adding 1 mol/L HCl to each well and wells were mixed gently for 30 s. Optical densities were read at 450 nm using a microtitre plate reader within 30 min.

Average absorbance values (A_{450}) were calculated for each set of reference standards, controls and samples. Each measurement was performed in duplicate and repeated twice for each sample. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration on a linear graph, with $OD_{standard}/OD_0\%$ ($B/B_0\%$) on the vertical (y) axis and concentration on the horizontal (x) axis. Results are expressed as ng/mL according to the calibration curve.

Detection of thymus cytokine mRNAs

Extraction of total RNA

Approximately 1 g of tissue from each thymus was stored at −70 °C until use. Frozen samples were ground in liquid nitrogen with a mortar and pestle and total RNA was extracted from the ground tissue using TRIZOL reagent (Invitrogen). Each RNA pellet was dissolved in 50 μL RNase-free, ultra-purified water and stored at −70 °C until use.

Primer design

Primers for detection of cytokine mRNA were designed using Primer Premier Software Version 5.0 based on porcine cytokine and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequences from GenBank. Primers were specific for each target mRNA (Table 1) and were used for both quantitative real-time PCR and one-step reverse transcriptase (RT)-PCR.

Plasmid preparation

One-step RT-PCR and the primers described above were used to generate plasmids encoding individual cytokines, cytokine receptors or GAPDH mRNA for generation of standard curves. RT-PCR products were purified using the TaKaRa Agarose Gel DNA Purification Kit Version 2.0 (TaKaRa). Individual purified PCR products were inserted into the pGEM-T Easy vector (Promega). Recombinant clones were transformed into *Escherichia coli* DH5α high-efficiency competent cells. White colonies were selected from X-Gal/isopropyl-β-D-1-thiogalactopyranoside (IPTG) ampicillin agar plates and grown in Luria–Bertani/ampicillin liquid media. Plasmids were purified using the TaKaRa MiniBest Plasmid Purification Kit Version 2.0 (TaKaRa). Ten-fold serial dilutions of individual recombinant plasmids were used for construction of standard curves using the cycle threshold (Ct) values obtained against the known concentration of serially diluted standard plasmids.

Thymus-generated cytokines and GAPDH were determined using spectrophotometric readings and converted to molecular copies using the following formula:

$$Y \text{ molecules}/\mu L = X \text{ g}/\mu L/(\text{transcript length in bp} \times 320) \times 6.02 \times 10^{23}$$

Relative quantification by SYBR Green I-based real-time RT-PCR

One-step RT-PCR (Quantitect SYBR Green PCR kit, TaKaRa) and the Icycler IQTM real-time detection system (Bio-Rad) were used to quantify mRNA expression. The RT-PCR conditions were as follows: 30 min at 50 °C, 3 min at 95 °C, 40 cycles of 30 s at 94 °C, 45 s at 58 °C and 30 s at 72 °C, then 8 s at 80 °C. To verify that only the specific product was amplified, melting point analysis was performed after the final PCR cycle. A single product at a specific melting temperature was obtained for each target. To compare the amplified products, signals were quantified using a standard curve with GAPDH as a control. The Ct was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. The copy numbers were calculated based on the standard curve for each individual plasmid. All mRNA samples were normalised using the following formula:

$$\text{Relative quantification of mRNA} = \text{copy of cytokine mRNA/copy of GAPDH mRNA}$$

Statistical analysis

Statistical analysis was performed using SPSS Version 11.5. Differences between each group were assessed using one-way analysis of variance (ANOVA). Correlation analyses used a one-tailed test. Values were considered significantly different when $P < 0.05$. All data are expressed as the mean ± *SD*.

Results

Changes in serum cytokine concentrations during transportation

Serum cytokine concentrations at 1, 2 and 4 h following transportation are shown in Table 2. IL-2 concentrations were highest at 1 h and decreased at 2 and 4 h. After 4 h of transportation, the concentration of IL-2 was significantly decreased ($P < 0.05$) compared with 1 and 2 h. Serum IL-6 followed a similar pattern, with a peak at 1 h, followed by a decrease. After 4 h of transportation, the serum concentration of IL-6 had decreased by 2.4-fold ($P < 0.05$) compared with the control value and by 3.2- and 2.7-fold compared to the 1 and 2 h time points, respectively ($P < 0.01$). Serum IL-10 concentrations were highest after 1 h of transportation. After 4 h of transportation, serum IL-10 concentrations were significantly decreased ($P < 0.01$) compared with control, 1 h and 2 h levels. Serum concentrations of IL-1β, TNF-α and IL-4 were not significantly altered by transportation.

Changes in IL-2 and IL-2R mRNA expression in the thymus during transportation

The results for thymic IL-2 and IL-2R mRNA expression are presented in Fig. 1a. IL-2 mRNA in the thymus was highest after 1 h of

Table 2
Changes in serum concentrations of cytokines in pigs during transportation stress.

Cytokine (ng/mL)	Period of exposure to transport stress (h)			
	0 (control)	1	2	4
IL-1β	3.67 ± 0.46	3.83 ± 0.46	3.70 ± 0.38	3.60 ± 0.33
IL-2	1.46 ± 0.21^{ab}	1.88 ± 0.34^{b}	1.69 ± 0.24^{b}	1.24 ± 0.21^{a}
IL-4	0.48 ± 0.03	0.41 ± 0.07	0.45 ± 0.08	0.47 ± 0.04
IL-6	0.70 ± 0.19^{b}	0.93 ± 0.15^{bB}	0.89 ± 0.24^{bB}	0.29 ± 0.04^{aA}
IL-10	1.63 ± 0.18^{bB}	1.83 ± 0.21^{bB}	1.76 ± 0.21^{bB}	1.10 ± 0.10^{aA}
TNF-α	0.14 ± 0.01	0.14 ± 0.03	0.14 ± 0.05	0.13 ± 0.05

IL, interleukin; TNF, tumour necrosis factor.
Values are the mean ± *SD*; $n = 5$ in each transport group, $n = 4$ in the control group. Capital and small letters indicate significant differences at $P < 0.01$ and $P < 0.05$, respectively.

Table 1
Primer sequences (5′–3′) used for detection of mRNAs specific for cytokine and housekeeping genes by real-time-PCR in pigs.

Target	Accession number	Sense primer	Antisense primer	Amplicon
IL-2	X56750	CTGGATTTACAGTTGCTTTTG	AGTCAGTGTTGAGTAGATGCTTT	347 bp
IL-6	M80258	GGCTGCTTCTGGTGATGG	AGAGATTTTGCCGAGGATGTA	146 bp
IL-10	L20001	GCATCCACTTCCCAACCA	GCAACAAGTCGCCCATCT	108 bp
IL-2R	U78317	CACGAATCTTTGAAGCGAGT	CCTGGTTGGTTAGGAAGTTTTAGTC	255 bp
IL-6R	AF147881	CGCAGGCACCTACCACTA	GCTGTCCCAAGGAATACGG	246 bp
IL-10R	AB116562	GTGGGACTCACCTGCTTT	TGTGGTCACCGTACTTGG	140 bp
GAPDH	AF017079	GAAGGTCGGAGTGAACGGAT	CATGGGTAGAATCATACTGGAACA	149 bp

IL, interleukin; IL-2R, interleukin-2-receptor; IL-6R, interleukin-6-receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.