Livestock Science 167 (2014) 323–330

Contents lists available at ScienceDirect

Livestock Science

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

Response to lipopolysaccharide in salivary components and the submandibular gland of pigs

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ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form
5 June 2014
Accepted 7 June 2014

Keywords:
Saliva
Submandibular gland
Lipopolysaccharide
Biomarkers
Pig

ABSTRACT

The diagnostic use of saliva for disease detection and health monitoring is increasing in recent years. To investigate whether the components of saliva change with lipopolysaccharide (LPS) challenge and clarify whether these changes directly relate to the submandibular gland (SMG), 12 pigs were employed in the present study. At 6 h after LPS intramuscular injection, samples including saliva, plasma and SMG were taken. The amylase, cortisol, Immunoglobulin A (IgA), tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) contents were analyzed by colorimetry, radioimmunoassay or ELISA, respectively. Chromogranin A (CgA) and lactoferrin protein concentrations were detected by western blot. Adenosine triphosphate (ATP) was measured by high-pressure liquid chromatography (HPLC) and the related gene expression was analyzed by reverse transcription polymerase chain reaction (RT-PCR) in SMG. The results showed that LPS treatment increased amylase activity and cortisol concentrations, whereas IL-1β, CgA and lactoferrin concentrations showed a decrease in saliva. Toll-like receptor 2 (TLR2), IL-1β and TNF-α gene expression in SMG increased. Decreased ATP concentration in SMG may be the reason for the opposite changes of IL-1β, lactoferrin and CgA content in the saliva and the SMG. The present study for the first time comprehensively demonstrates that the altered parameters in saliva can indicate the body inflammation in pigs, and they may become useful, noninvasive biomarkers for the evaluation of body state.

1. Introduction

In recent years interest in using saliva as a diagnostic sample for disease detection and health monitoring is increasing because it is non-invasive, easily accessible and contain high information (Sexton et al., 2011; Yin et al., 2012; Gutiérrez et al., 2013a, 2013b). Although saliva is mainly composed of water, it also consists of enzymes, ions, hormones and immune markers produced mostly by the salivary glands (Pedersen et al., 2002). Salivary cortisol is the commonly used biomarker of psychological stress (Hellhammer et al., 2009), and some components in saliva such as α-amylase (Nater et al., 2006), β-endorphin (Pikula et al., 1992) and Immunoglobulin A (IgA) (Moreira et al., 2011) have been shown to respond to biological stress.

Bacterial lipopolysaccharide (LPS), the primary glycolipid component of the outer membrane of gram-negative bacteria, plays a key role as a determinant of bacterial interactions with the host. Several studies have reported that LPS administration could increase the pro-inflammatory cytokines action in plasma such as tumor necrosis factor alpha (TNF-α) (Webel et al., 1997; Wright et al., 2000), interleukin 6 (IL-6) (Webel et al., 1997, 1998), interleukin 1 beta (IL-1β) and interferon-γ (Armstrong and Spears, 2003). In the saliva, IL-1β protein was shown to increase in response to an endotoxin injected intraperitoneally in mice (Yao et al.,

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http://dx.doi.org/10.1016/j.livsci.2014.06.013
1871-1413/

2005). However, most of the components changes in saliva are still largely unknown when the animal is injected with LPS.

The salivary gland has three major glands (parotid, submandibular and sublingual), that are all innervated by both sympathetic and parasympathetic nerves. The submandibular gland (SMG) mainly produces not only a large number of nerve growth factors, epidermal growth factors, renin and kallikreins, but also some antimicrobial proteins (Abad Garrido and Garcia Ballesta, 1985; Sabbadini and Berczi, 1995). Chromogranin A (CgA) is an acidic, hydrophilic glycoprotein that is co-released with epinephrine or norepinephrine in the adrenal medulla (Pieroni et al., 2007). The expression and localization of CgA have been shown to be in human SMG (Saruta et al., 2005), and it is stored in the granular convoluted tubule of SMG (Kanno et al., 1999). The concentrations of CgA in both the saliva and blood have been shown to elevate in people under stress (Nakane et al., 2002). Lactoferrin is an iron-binding and multifunctional glycoprotein of the transferrin family (Garcia-Montoya et al., 2012) and can be secreted by intercalated duct cells of the salivary glands (Waszkiewicz et al., 2012). It can interact with the LPS of gram-negative bacterial membranes showing antimicrobial activity in the innate immune response (Farnaud and Evans, 2003; Cuenca et al., 2013). However, whether CgA and lactoferrin both in saliva and the SMG changed after LPS injection is unclear.

Therefore, in the present study we used growing pigs as animal models to analyze the cortisol, amylase, pro-inflammatory cytokines, CgA and lactoferrin contents in saliva after LPS infection and detect the related changes in the SMG to further clarify the possible mechanism. The results will provide new information for the non-invasive biomarkers research.

2. Materials and methods

2.1. Animals and experimental design

The slaughter and sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China. The experimental protocol was specifically approved by the Animal Ethics Committee of Nanjing Agricultural University. Twelve male growing piglets (Large White × Landrace × Duroc) with an average body weight of 12 ± 0.5 kg were randomly selected from Huaian commercial farm and randomly divided into two groups. Before the experiment, they were adapted for 7 days with ad libitum and free access to water. During the 7 adaptation days, the pigs were trained to be accustomed to saliva sampling. Before the LPS injection the pigs were fasted for two hours. The LPS from *Escherichia coli* serotype K-235 (phenol extracted) (Sigma-Aldrich Ireland Ltd., Dublin, Ireland), was dissolved in 0.9% NaCl solution. Pigs were injected intramuscularly (i. m.) with 2 ml of saline (0.9% NaCl) or 2 ml 15 μg/kg body weight (BW) LPS at 09:00. Six hours after treatment with LPS, saliva was collected by the following steps. Firstly, medical cotton was tied with 100 cm string and then, the string was shaken in front of the pigs in order to attract their attention until bitten. After 5 min, the cotton was recovered by withdrawing the string. Approximately 8 ml of saliva supernatant was collected by centrifugation at 3500*g* and at 4 C for 10 min, and was stored at −80 °C until analysis. After taking saliva, pigs were sacrificed. Consequently, the blood was collected with anticoagulant tubes containing heparin (Nanjing Jiancheng Bioengineering Institute, China) and the submandibular gland was sampled and stored at −80 °C until analysis.

2.2. Body temperature analysis

A veterinary infrared non-contact thermometer (HAORUNQI, China) was used to determine body temperature which was recorded by measuring 6–8 cm behind the pig's ear. Body temperature was recorded at 1 h before and 1, 2, 3, 4 and 6 h after injection with LPS or saline.

2.3. Plasma and saliva analysis

Cortisol and TNF-α concentration in plasma and saliva were measured using a commercial multispecies ^{125}I radioimmunoassay (RIA) Kit (Beijing North Institute of Biological Technology, China) with assay sensitivities of 2 ng/ml and 6 fmol/ml, respectively. The intra- and inter-assay variations were 10 and 15%, respectively, for both kits. Plasma and saliva concentrations of IgA and IL-1β were determined using a commercial ELISA Kit (Bethyl Laboratories Inc, Abcam) according to the manufacturer's instructions. The detection limits were 1.37 ng/ml and 6 pg/ml, respectively. The intra- and inter-assay coefficients of variations were 10% and 12%, respectively. The salivary amylase concentration was determined by enzymatic colorimetric methods using a commercial kit (Nanjing Jiancheng Bioengineering Institute). The detection limit was 104.5 U/dl, the intra- and inter-assay coefficients of variations were 2.5% and 4.56% respectively.

2.4. Total RNA isolation, quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated from homogenized SMG with the Trizol RNA Kit (Invitrogen Life Technologies, USA). The concentration of RNA was measured by the absorbance at 260 nm by using an Eppendorf BioPhotometer (Gene Company Ltd., China). Total RNA was reverse-transcribed following a series of protocols including 12 μM random primer (6-mer) (TaKaRa, Biotechnology Co., China), 100 U Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Shanghai, China), 10 U RNase inhibitor (Promega, Shanghai, China), 10 mM deoxy-ribonucleoside triphosphate (dNTP) mixture. The PCR reactions were carried out with 2 μL reverse transcribed (RT) products, 0.2 μM primers, 8.5 μL triple-distilled water and 12.5 μL SYBR Green Real-time PCR Master Mix (TOYOBO Ltd., Japan). The primers were designed and synthesized by Takara Biotechnology (Table 2).