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Differential proteomic profiling of endometrium and plasma indicate the importance of hydrolysis in bovine endometritis

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ABSTRACT

Endometritis is an important disease of dairy cows that leads to significant economic losses in the dairy cattle industry. To investigate the alteration of proteins associated with endometritis in the dairy cow, the isobaric tags for relative and absolute quantification (iTRAQ) technique was applied to quantitatively identify differentially expressed proteins (DEP) in the endometrium and peripheral plasma of Chinese Holstein cows with endometritis. Compared with the normal (control) group, 159 DEP in the endometrium and 137 DEP in the plasma were identified in cows with endometritis. Gene ontology analysis demonstrated that the predominant endometrial DEP were primarily involved in responses to stimulus and stress processes and mainly played a role in hydrolysis in the extracellular region. The predominant plasma DEP were mainly components of the cytosol and non–membrane-bound organelles, and they were involved in the response to stress and regulation of enzyme activity. Protein–protein interaction of tissue DEP revealed that some core seed proteins, such as RAC2, ITGB2, and CDH1 in the same network as CD14, MMP3, and MMP9, had important functions in the cross-talk of pathways related to extracellular proteolysis. In summary, significant enzymatic hydrolase activity in the extracellular region is proposed as a molecular mechanism by which altered proteins may promote inflammation and hence endometritis.

Key words: isobaric tags for relative and absolute quantification (iTRAQ), bovine endometritis, differentially expressed proteins, hydrolysis

INTRODUCTION

Uterine infection and endometritis are important causes of infertility and reproductive losses in dairy cattle worldwide (Gilbert, 2011; LeBlanc, 2014). The protective physical barriers to contamination provided by the uterine luminal epithelium, cervix, vagina, and vulva are all disrupted during normal parturition (Sheldon and Dobson, 2004). This disruption and the large volumes of fluid and tissue debris in the uterine lumen both allow bacteria from the environment or the animal's feces to contaminate the uterus postpartum (Bondurant, 1999; Sheldon and Dobson, 2004). Several studies showed the presence of bacteria in the uterus of more than 90% of cows within the first 2 wk after calving (Dohmen et al., 1995; Sheldon et al., 2008). Although most bacterial contaminants are gradually cleared from the uterus via uterine involution and innate immune response (Azawi, 2008; Singh et al., 2008), up to 20% of cows have clinical metritis and 20% to over 50% of cows subsequently develop subclinical inflammation of the uterus (endometritis) beyond 3 wk postpartum (Sheldon et al., 2009a; LeBlanc, 2014). Endometritis causes uterine tissue damage, embryonic death and early abortion, delayed onset of ovarian cyclicity, extended luteal phases, and reduced conception rates in affected cattle (Sheldon et al., 2009b; Gilbert, 2011). These problems lead to infertility and substantial financial losses in the dairy industry (Sheldon et al., 2009a).

The endometrium has crucial roles in female reproduction including the regulation of the estrous cycle, sperm transit, nourishment of the early embryo, and formation of the placenta. Furthermore, the endometrial luminal epithelial cells and the underlying stromal cells express pathogen recognition receptors and mount an innate immune response to microbes or microbial ligands (Hickey et al., 2011; Oguejiofor et al., 2015a,b). The major bacteria isolated from cows with endometritis include *Escherichia coli* and *Trueperella pyogenes*, followed by other anaerobic species, such as *Fusobacte-*

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rium and *Bacteroides* (Dohmen et al., 2000; Williams et al., 2005; Bicalho et al., 2012). Although the effects of different types of bacteria on uterine disease mechanisms are not completely understood (Westermann et al., 2010), unregulated inflammation is known to lead to disease (Maybin et al., 2011). This outcome can impair reproductive performance not only during the infection but also after the clinical signs of endometritis have resolved (Plöntzke et al., 2010; LeBlanc, 2014). Interestingly, some evidence suggests that certain viruses such as bovine viral diarrhea virus (Grooms, 2004; Oguejiofor et al., 2015a; Cheng et al., 2016) and bovine herpes virus-4 (Donofrio et al., 2009) contribute to the etiology of uterine disease.

Endometritis is described histopathologically as a superficial inflammation of the endometrium, with histological evidence of inflammation (Sheldon et al., 2006). Endometritis has been defined based on the presence of abnormally increased PMN in uterine luminal fluid (Kasimanickam et al., 2004; Gilbert et al., 2005) or the biopsy of endometrial tissue (Galvão et al., 2011). In addition, increased endometrial expression of inflammatory genes may be present, including genes coding for granulocyte chemotactic protein 2, IL-1β and IL-8, and tumor necrosis factor (**TNF**) (Fischer et al., 2010; Kasimanickam et al., 2014). Elevated levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10) in serum have also been used to identify the development of bovine endometritis (Islam et al., 2013; Kasimanickam et al., 2013). Previously, genome-wide transcriptomic profiling of bovine endometrium using mRNA-Seq showed that immune activation and inflammation preceded tissue proliferation and repair in the healthy postpartum endometrium (Foley et al., 2012). This finding highlighted the importance of a balanced inflammatory immune response as key to sufficient bacterial clearance and restoration of an endometrial environment capable of supporting a new pregnancy (Jabbour et al., 2009). Limited studies exist on the bovine uterine proteome. One study identified 9 proteins using 2-dimensional (**2D**) electrophoresis to compare the proteins present in the uterine luminal fluid between pregnant and nonpregnant animals (Ledgard et al., 2009). Another study used 2D fluorescence difference in-gel electrophoresis to compare the proteome of the pregnant and nonpregnant endometrium (Berendt et al., 2005). However, scant information is available on the proteomic profile of cows with endometritis. A previous study identified 14 endometritis-associated proteins using 2D electrophoresis (Choe et al., 2010).

Recent developments in the field of proteomics have led to a renewed interest in animal disease diagnosis and treatment. Isobaric tags for relative and absolute quantification (**iTRAQ**) is the most popular technique, and combined with multidimensional liquid chromatography (**LC**) and tandem MS, it is used to study differentially expressed proteins (**DEP**). The aim of this study was to use the iTRAQ technique to characterize the proteomic changes in endometrial tissue and plasma from Chinese Holstein dairy cows with endometritis. The bioinformatics analysis of the differential proteome may enable a new understanding of the main proteins or pathways associated with bovine endometritis and provide a foundation for future diagnosis and treatment of diseased animals.

MATERIALS AND METHODS

Animals and Collection of Samples

Sample collection was carried out under license in accordance with national guidelines (Ministry of Agriculture of China 2015/No. 18). Rectal palpation and cervico-vaginal mucus observation were conducted to identify normal animals or cows with endometritis. Endometrial biopsies and blood specimens were collected from Chinese Holstein cows, aged from 2 to 5 yr, at 21 to 35 d postpartum. Ten candidate cows had obvious signs of clinical endometritis (i.e., presence of purulent or mucopurulent vaginal discharge), but they did not show any symptoms of other local or systemic diseases beyond the focus of this study. Another 10 cows without signs of endometritis were categorized as the normal control animals. Briefly, 5 mL of blood was collected from the jugular vein into commercial vacuum tubes and thoroughly mixed with the anticoagulant $EDTAK_2$. This samples were centrifuged at 2,500 × *g* for 15 min, and 0.5 mL of plasma was recovered and quickly frozen with liquid nitrogen. Endometrial biopsies were quickly frozen in liquid nitrogen and fixed in 4% formalin, respectively. After formalin fixation, tissue specimens were embedded in paraffin blocks using routine procedures, followed by hematoxylin and eosin staining and histopathological examination under the microscope to identify pathological state.

Protein Isolation, Digestion, and Labeling with iTRAQ Reagent

Protein extraction was performed on 4 nonendometritis samples and 4 endometritis samples that were selected according to histopathological results. Briefly, the frozen tissue was ground into powder and then dissolved in lysis buffer I (7 *M* urea, 2 *M* thiourea, 4% propanesulfonic acid, 40 m*M* Tris-HCl, pH 8.5) containing complete protease inhibitor. The cells were lysed by