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Microarray-based gene expression profiling of peripheral blood mononuclear cells in dairy cows with experimental hypocalcemia and milk fever

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ABSTRACT

Although a molecular diagnostic assay using clinically accessible tissue, such as blood, would facilitate evaluation of disease conditions in humans and animals, little information exists on microarray-based gene expression profiling of circulating leukocytes from clinically hypocalcemic cows. Therefore, peripheral blood mononuclear cells from dairy cows with experimentally induced hypocalcemia or spontaneous milk fever were subjected to oligo-microarray analysis to identify specific biomarker genes. In experimental hypocalcemia induced by a 4-h infusion of 10% disodium EDTA (n = 4), 32 genes were significantly up- or downregulated compared with control treatment (4-h infusion of 11% calcium EDTA; n = 4). In cows with milk fever (n = 8), 98 genes were expressed differentially (either up- or downregulated) compared with healthy parturient cows (n = 5). From these data, the following 5 genes were selected as being strongly related to both experimental hypocalcemia and milk fever: protein kinase (cAMP-dependent, catalytic) inhibitor β (*PKIB*); DNA-damage-inducible transcript 4 (*DDIT4*); period homolog 1 (*PER1*); NUAK family, SNF1-like kinase, 1 (*NUAK1*); and expressed sequence tag (*BI537947*). Another gene (neuroendocrine secretory protein 55, *NESP55*) was also determined to be specific for milk fever, independently of hypocalcemia. The mRNA expression of these 6 genes in milk fever cases was verified by quantitative real-time reverse-transcription PCR and was significantly different compared with their expression in healthy parturient cows. In the present study, the selected genes appeared to be candidate biomarkers of milk fever because the continuous interactions between blood cells and the entire body suggest that subtle intracellular changes occur in association with disease. However, before any genomic biomarkers are incorporated into clinical evaluation of the disease, the effect of hypocalcemia on the mRNA expression of these genes in the tissues that regulate calcium homeostasis in dairy cows should be determined.

Key words: dairy cow, microarray-based gene expression profiling, milk fever, peripheral blood mononuclear cell (PBMC)

INTRODUCTION

Milk fever is an afebrile disease of primarily multiparous cows that occurs around the time of parturition. Affected cows show characteristic clinical signs, such as progressive neuromuscular dysfunction with flaccid paralysis, recumbency, circulatory collapse, cold extremities, depressed consciousness, and coma (Goff, 2009). The biochemical characteristic of this disease is severe hypocalcemia, which most likely explains the associated clinical signs (Goff, 2009). Investigation of this disease has focused on blood Ca concentrations around parturition because of the close relationship between hypocalcemia and the onset of milk fever (Goff, 2009). Several other blood parameters, such as phosphorus, magnesium, parathyroid hormone, calcitonin, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], and some bone metabolic markers associated with the regulation of Ca metabolism have also been considered in an attempt to further understand Ca homeostasis and the pathophysiology of milk fever (Liesegang et al., 1998; Larsen et al., 2001; Kim et al., 2010).

Microarray technology has recently been shown to be a useful tool for simultaneously measuring the expression of thousands of genes and for clarifying the molecular mechanisms of many diseases in humans and animals. In cattle, this molecular genomic approach has been utilized to study the pathophysiology of lameness (Almeida et al., 2007), ketosis (Loor et al., 2007), mastitis (Lutzow et al., 2008), brisket disease (Newman et al., 2011), and growth retardation (Ishida et al., 2013). In most experiments, this technique was applied to genes within various body tissues (e.g., liver, kidney, intestine, mammary gland, or lymph node) from diseased animals, whereas other studies have used genes from

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circulating leukocytes to identify biomarkers of pathophysiological status (Almeida et al., 2007; Newman et al., 2011; Ishida et al., 2013). Although still in the early stages of research and development, genomic biomarkers can provide comprehensive insight into pathophysiological processes as well as more precise prediction of disease outcomes not previously attainable using protein or biochemical biomarkers (Ginsburg and Haga, 2006). In addition, a molecular diagnostic assay using clinically accessible tissue, such as blood, would facilitate evaluation of disease conditions of animals (Almeida et al., 2007). To our knowledge, little information is available on microarray-based gene expression profiles of circulating leukocytes from hypocalcemic dairy cows with milk fever.

In the present study, peripheral blood mononuclear cells (**PBMC**) from dairy cows with experimentally induced hypocalcemia or spontaneous milk fever were subjected to oligo-microarray analysis. Genes that showed common altered expressions in cows with experimentally induced hypocalcemia and spontaneous milk fever were then assessed by quantitative real-time reverse-transcription PCR (**q-PCR**). The aim of this study was to identify potential biomarkers of hypocalcemia and milk fever in PBMC of dairy cows by microarray-based gene-expression profiling.

MATERIALS AND METHODS

This animal experimental protocol was approved by the Iwate University Laboratory Animal Care and Use Committee.

Experimentally Induced Hypocalcemia

Four healthy, nonpregnant, nonlactating, ovariectomized Holstein cows (6.3–8.3 yr of age, 520–665 kg of BW) were used. A bilateral ovariectomy was performed in each cow by lateral laparotomy at least 3 mo before the experiment to exclude the effects of gonadal steroid hormones (Devkota et al., 2012). The cows were housed individually and loose-tied at least 4 wk before the start of the experiment. The cows were fed 5 kg of grass hay and 0.5 kg of concentrate twice daily and had access to tap water ad libitum.

One hundred grams of disodium EDTA (Na_2-EDTA; Kanto Chemical, Tokyo, Japan) was dissolved in 1,000 mL of endotoxin-free sterile distilled water and adjusted to pH 7.0 with sodium hydroxide. One hundred ten grams of Ca-EDTA (Dojindo, Kumamoto, Japan) was dissolved in 1,000 mL of endotoxin-free sterile distilled water and adjusted to pH 7.0 with hydrochloric acid. The solutions were transferred to a sterile container through a 0.2-μm filter (Sterifix; Forte Grow Medical, Tochigi, Japan) to remove bacteria.

The experiment was performed according to a 2 × 2 crossover design with 2-wk intervals. In brief, an intravenous catheter (UK catheter kit, 14 gauge, 30 cm; Unitika, Osaka, Japan) was inserted into each jugular vein 1 d before the EDTA infusion to alleviate the stress associated with the infusion and blood collection. Cows were infused with either Na_2-EDTA solution (hypocalcemic treatment) or Ca-EDTA solution (control treatment) at a rate of 0.25 mmol/kg per minute for 4 h, based on previous studies (Jørgensen et al., 1999; Mellau et al., 2004). The animals were monitored for clinical signs of hypocalcemia (e.g., dry nose, staggering, astasia, reduced ruminal activity, increased heart rate, increased respiratory frequency, sweating, and ear coldness). The infusion was suspended temporarily when ataxia was observed, and was resumed when the affected cow regained a standing posture.

Heparinized blood collection was performed immediately before infusion (0 h; pretreatment) and at 1, 2, 3, 4, 6, 8, 10, 12, 18, and 24 h after the start of infusion. All blood samples were placed on ice and transported to the laboratory within 30 min. All blood samples were used to measure plasma Ca levels, and blood at 0, 4, and 24 h was used for PBMC gene expression analysis.

Spontaneous Milk Fever

A total of 13 Holstein dairy cows, including 8 parturient cows (5.2–8.7 yr of age) diagnosed with milk fever within 2 d after parturition and 5 clinically healthy parturient cows (group C; 5.7–8.5 yr of age) within 2 d after parturition, were used. The clinical diagnosis of milk fever was determined based on the clinical history and presence of clinical signs characteristic of hypocalcemia, as reported previously (Sasaki et al., 2013). All cows showed clinical hypocalcemia with <1.4 mmol/L of Ca in the blood samples obtained before the first treatment and were treated with an intravenous infusion of 20% Ca borogluconate solution (500 mL). The milk fever cases were divided into 2 categories according to the response to the first Ca treatment: the immediate-response group, in which cows could stand within 1 d after a single Ca treatment (group A, n = 4), and the group requiring further treatment after 1 d (group B, n = 4).

Heparinized blood samples were collected via the jugular vein immediately before the first Ca treatment in groups A and B, and within 2 d postpartum in group C, for plasma biochemistry and gene expression evaluation. All samples were placed on ice and transported to the laboratory within 3 h.