Blood ionized calcium levels and acute-phase blood glucose kinetics in goats after intramammary infusion of lipopolysaccharide

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ABSTRACT. The aim of this study was to determine the blood ionized calcium (Ca) levels and acute-phase blood glucose kinetics in goats with mastitis induced by an intramammary challenge of lipopolysaccharide (LPS). Five goats were subjected to intramammary challenge of either LPS (10 µg) or saline (control). Some clinical manifestations (rectal temperature, pulse rate, respiration rate, ruminal motility, physical activity, and dehydration) were observed, and blood was collected for the measurement of several parameters [ionized and total Ca levels, blood glucose level, pH, and white blood count (WBC)] at 0 (just before challenge), 1–4, 6, 8, 12 and 24 hr post-challenge in both the LPS and control phases. Milk was collected at 0 (just before challenge), 4, 8, 12 and 24 hr post-challenge to measure the somatic cell count (SCC) and N-acetyl-beta-D-glucosaminidase (NAGase) activity. In the LPS phase, increased rectal temperature, significantly decreased ionized Ca and total Ca levels and WBCs were observed compared with those at 0 hr, although there were no differences in all parameters between phases. LPS infusion significantly increased SCCs in milk and NAGase activity. The present results demonstrated that, during the acute phase of mastitis induced by intramammary challenge by LPS at a concentration sufficient to cause general symptoms in goats, a decreased blood ionized Ca level occurs, but not hypoglycemia.

KEY WORDS: glucose, goat, ionized calcium, lipopolysaccharide, mastitis

Mastitis is not only a bovine disease, which is a global issue, but also an important challenge for public health [14]. Acute coliform mastitis (ACM) is one form of mastitis, for which Escherichia coli is a major pathogen, and it is associated with a problematically high culling rate because its general symptoms can be serious. ACM progresses rapidly, making early detection and treatment vital. Early treatment strategies for ACM are based on an understanding of the state of the disease in the acute phase, and such understanding has thus assumed much significance. Serious general symptoms of ACM are induced through a biological reaction to the lipopolysaccharide (LPS) present in the cell wall of the pathogen, involving the release of inflammatory mediators [1]. Until now, mastitis has been studied in disease models induced by intravenous LPS challenge. These models have shown an acute-phase reaction characterized by hypocalcemia [18] and hypoglycemia, which follows a transiently high blood glucose level [10].

The blood ionized calcium (Ca) level is a clinical marker of circulating, physiologically active Ca. Ionic hypocalcemia is reportedly used as a prognostic indicator in dogs [4], in which it can occur with endotoxemia [3]. In bovine ACM, an association with hypocalcemia is reportedly much more likely when the condition is severe rather than mild [20]; however, acute-phase plasma ionized Ca levels have not been fully investigated. It has recently become clear that acute endotoxemia-induced hypoglycemia occurs when immunological glucose clearance overcomes the whole body’s glucose regulation mechanism [10]. Studies have stressed the importance of maintaining blood glucose levels in people with septic shock. Accordingly, an understanding of blood glucose kinetics in the acute phase of ACM is vital for the development of evidence-based therapies for this condition.

Serious symptoms are not been observed in every clinical case of ACM, and the symptoms can vary from mild to severe [19]. Contrasting evidence has emerged from reports on ACM up to the present: cases with no development of endotoxemia have been seen [11], while other researchers have noted endotoxemia in approximately 50% of ACM cases [7]. We consider that the disparity between these reports may reflect differences in the severity of ACM, although no consensus has emerged on this issue. Until now, models of ACM have been induced by intravenous LPS challenge, and they have attempted to reproduce a severe disease state...
with accompanying endotoxemia, and mild ACM has not been investigated. Consequently, there is little understanding of blood ionized Ca levels and acute-phase blood glucose kinetics in milder forms of mastitis induced with weak LPS inoculation.

In this study, the aim was to determine blood ionized Ca levels and acute-phase blood glucose kinetics in goats with mild mastitis induced by intramammary inoculation with LPS.

**MATERIALS AND METHODS**

The study was approved by the ethics committee of Hiroshima University (No. C14-5).

**Experimental design**

A single-case design was used to assess five goats. All goats were Tokara species (17.8 to 25.0 kg; 67 to 120 days in milk; parity 1 to 3) and clinically healthy. Their udders were free from mastitis pathogens and had a low milk somatic cell count (SCC) (<150,000 cells/ml) before the experiment. Treatments were sterile isotonic saline (0.9% NaCl) as a control or LPS inoculation to the left mammary gland.

**Lipopolysaccharide and intramammary challenge**

The LPS (Escherichia coli O111; Wako Pure Chemical, Osaka, Japan) challenge was done with 10 µg diluted into 5 ml of sterile isotonic saline (0.9% NaCl). The dose of LPS was selected based on a previous study [16]. This LPS dose is considered to be a medium dose and was chosen because it introduces a systemic and stereotypic clinical response in LPS-treated cows. As a control, one mammary gland of each goat was infused with saline. The time of infusions was designated 0 hr.

**Clinical observations**

Systemic and local signs were recorded throughout the experiment at 0–4, 6, 8, 12 and 24 hr post challenge. Rectal temperature, heart rate, respiratory, rumen contraction rate, general attitude, and dehydration status were evaluated as systemic signs.

**Sampling and analytical procedures**

Blood samples were collected from the jugular vein in disposable syringes with no anticoagulant, vacutainer plain and EDTA tubes (Venoject®, Terumo Corp., Tokyo, Japan), at 0 (immediately before challenge), 1–4, 6, 8, 12 and 24 hr after challenge. Whole blood with the syringe was analyzed with the handheld i-STAT®1 analyzer and CG8+ cartridges (Abbot Laboratories, Princeton, NJ, U.S.A.) immediately. The CG8+ cartridges provided values for sodium (Na mmol/l), potassium (K mmol/l), ionized calcium (iCa mmol/l), glucose (Glu mg/dl), hematocrit (Hct% packed cell volume), pH, partial pressure of carbon dioxide (PCO₂ mmHg), partial pressure of oxygen (PO₂ mmHg), and total concentration of carbon dioxide (TCO₂ mmol/l). Ionized calcium concentrations were pH-corrected by the following equation (corrected iCa=actual iCa {1–0.53 (7.4-pH)}) [15]. Serum was separated by centrifuging within 24 hr and stored at −70°C. Plasma total Ca concentration was measured using the AU480 Chemistry System (Beckman Coulter, Inc., Brea, CA, U.S.A.). Samples anticoagulated with EDTA were used for complete blood count analysis. Milk samples were collected from all mammary glands at 0 (immediately before challenge), 4, 8, 12 and 24 hr. Each milk sample was collected into sterilized tubes and stored until SCCs were determined using the DeLaval cell counter (DCC: DeLaval International AB, Tumba, Sweden) based on the method of Kawai et al. [8]. NAGase activity was determined using the β-N-Acetylglucosaminidase Assay Kit (Sigma-Aldrich Co., LLC., St. Louis, MO, U.S.A.). Milk samples were centrifuged at 3,000 rpm for 10 min at 20°C, and the resultant whey was provided to determine NAGase activity, which was calculated from the difference between the absorbance value of the whey sample and the absorbance value of the unreacted substrate of the same whey sample (background control) to avoid the effect of whey color.

**Statistical analysis**

SCC and NAGase activity were converted to common logarithm values. The paired t-test was used to compare each measured value before and after LPS challenge. Significance was considered at P<0.05. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (the R foundation for Statistical Computing, Vienna, Austria). This is a modified version of R commander that enables the application of statistical functions frequently used in biostatistics [6].

**RESULTS**

**Clinical observations**

All goats showed transient loss of appetite and signs of fever post-challenge, but they recovered, and all general symptoms disappeared by 24 hr post-challenge. Rectal temperature initially increased at 1 hr post-challenge, peaked at 4 hr post-challenge, and returned to normal by 24 hr post-challenge, when physical activity and appetite also showed recovery (Fig. 1). Rectal temperature at 2 to 12 hr was significantly higher in the LPS phase than in the control phase. The respiratory rate, pulse rate, and ruminal motility showed no differences from the control phase (data not shown).
Blood tests
In the LPS phase, blood ionized Ca levels decreased significantly from 2 to 4 hr post-challenge compared with at 0 hr (Fig. 2a), and blood total Ca levels decreased significantly from 3 to 6 hr post-challenge (Fig. 2b). The glucose level of one goat in the control phase was missing. Blood glucose levels were not different between the control and LPS phases (Fig. 2c). Blood Na, K, Hct, pH, pCO₂, pO₂ and TCO₂ also showed no differences between the two phases (data not shown). The WBCs decreased from 2 hr post-challenge and tended to recover from 12 hr post-challenge; however, they remained in a decreased state at 24 hr post-challenge (Fig. 2d).

Milk testing
SCCs were significantly increased at 4 hr post-challenge and remained at a high level until 24 hr (Fig. 3a). NAGase activity increased significantly at 8 hr post-challenge and continued to increase until 24 hr post-challenge (Fig. 3b).

DISCUSSION
After challenge with LPS, goats showed an increase in rectal temperature, which peaked at 4 hr post-injection, along with general symptoms including reduced appetite and physical activity. At 24 hr post-challenge, goats showed recovery of appetite and signs that their fever had subsided. These results demonstrated that mild LPS-induced mastitis with transient general symptoms was successfully reproduced.

Intramammary LPS challenge did not decrease ruminal motility in this study. An association between decreased ruminal motility and ACM severity has been reported in a bovine intramammary E. coli infection study [12], and decreased ruminal motility is suggested to reflect an effect of tumor necrosis factor (TNF) [17]. It is not possible to say whether the same phenomenon is observed in goats; however, any TNF effects triggered by LPS at the concentration injected in this study may have been slight.

Blood total and ionized Ca levels were transiently decreased after LPS challenge. Hypocalcemia is less likely in mild ACM than in moderate to severe ACM, although it is a common finding in severe cases [19]. Such reports were supported by the result for the blood total Ca level in the present study, which showed only a slight decrease. It was also found that the decrease in the blood total Ca level was preceded by that in ionized Ca, which showed a similar tendency thereafter. Accordingly, we consider that the decrease in the blood total Ca level resulted from that in ionized Ca. LPS suppresses gluconeogenesis [13], and intravenous LPS [10] and cytokine [9] challenge reportedly cause hypoglycemia after a transient increase in blood glucose levels. However, there were no changes in blood glucose levels in the present experiment; therefore, we consider that any endotoxemia and cytokinemia due to LPS at the concentration injected would not have been strong enough to affect the blood glucose level.

SCC and NAGase activity were both increased; however, the increase in SCC preceded that in NAGase activity, and the increases were not concomitant. At 24 hr post-challenge, SCC showed a shift to decrease, but NAGase activity showed the same increasing trend as at the previous time points. NAGase is a lysosomal glycosidase released into milk from epithelial cells in mammary tissue, and it reflects the destruction of mammary tissue and dissolution of inflammatory neutrophils [5]. The present results showed that damage to mammary tissue started to occur after an influx of neutrophils into the udders. We consider that the mammary tissue may have been damaged after infiltration of active neutrophils into the udders based on reports of mammary tissue damage due to neutrophil-mediated reactive oxygen and proteases in LPS-induced mastitis [21].

In this study, a goat intramammary LPS-induced mastitis model that manifested general symptoms was created, and blood total
Fig. 2. Ionized calcium concentration (a), total calcium concentration (b), glucose level (c) and white blood cells (d) in blood after intramammary challenge with LPS (red: n=5) and saline (blue: n=5). Values are means ± SEM. Asterisks indicates significant differences compared with at 0 hr.

Fig. 3. Somatic cell counts (a) and NAGase activity (b) in milk after intramammary challenge with LPS (red: n=5) and saline (blue: n=5). Values are means ± SEM. Asterisks indicates significant differences compared with at 0 hr.
and ionized Ca levels were slightly decreased without any effect on the blood glucose level. The present findings of a normal blood glucose level may indicate that this parameter is not affected by endotoxemia or cytokinemia. An association between ACM severity and cytokinemia has been identified [2], and we consider that blood ionized and total Ca levels measurement may be useful for diagnosing the severity of ACM in initial medical examinations.

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