Metabolic and Lactational Responses during Recombinant Bovine Tumor Necrosis Factor- α Treatment in Lactating Cows

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ABSTRACT

This study examined the effects of recombinant bovine tumor necrosis factor- α (rbTNF) administration on metabolic and hormonal responses and lactational performance in dairy cows. Twelve lactating Holstein cows were injected subcutaneously with rbTNF (2.5 μ g per kg per d) or saline (3 ml per head per d) at 1200 h daily for 7 d (d 0 – 6) and used in a crossover design. The rbTNF treatment induced increases in plasma haptoglobin, nonesterified fatty acid, cortisol, and growth hormone levels compared with the control levels. The rbTNF-treated cows had lower triiodothyronine and insulin-like growth factor-1 concentrations than control cows. In a somatotropin challenge on d 6, the somatotropin response to somatotropin (0.25 μ g/kg) was smaller in the rbTNF group than in the control. The rbTNF treatment also produced increases of the nitrite plus nitrate concentration in plasma and milk during the period between d 1 and 7. Milk yield was reduced by rbTNF administration from d 1 to 8. The percentage of milk fat was increased on d 1 – 7 by rbTNF treatment, but milk protein content in the rbTNF group was decreased on d 5 and 7 as compared with that in the control group. These results support the possibility that tumor necrosis factor- α is responsible for the changes in hormone secretion, milk production and composition, and inflammatory parameters observed during coliform mastitis.

(**Key words:** recombinant bovine tumor necrosis factor- α , metabolite, hormone, mastitis)

Abbreviation key: APR = acute phase response, HP = haptoglobin, IGF-1 = insulin-like growth factor 1, LPS = lipopolysaccharide, NEFA = nonesterified fatty acid, NO = nitric oxide, NO_x = nitrite plus nitrate, rbTNF = recombinant bovine tumor necrosis factor- α , ST = somatotropin, STRH = somatotropin releasing hormone, TNF = tumor necrosis factor- α , T₃ = triiodothyronine.

INTRODUCTION

Mastitis is recognized as one of the most important diseases affecting dairy cows worldwide (Hill et al., 1979). It can be caused by a large number of bacterial species, and in particular, *Escherichia coli*-induced mastitis with severe clinical symptoms is most frequently observed during early lactation (Hill et al., 1979). Coliform mastitis has adverse effects on the economics of milk production by reducing the quantity and quality of milk (Shuster et al., 1991c).

Bacterial lipopolysaccharide (LPS), often called endotoxin, is considered to be the key molecule in the development of coliform mastitis (Shuster et al., 1991a). Indeed, intramammary infusion of LPS in dairy cows induces clinical (fever, tachycardia), metabolic (a decrease in plasma Zn and Fe concentrations), and hematologic (neutropenia followed by neutrophilia) disturbances, which are a set of responses generally referred to as the acute phase response (APR) (Lohuis et al., 1992). In these cows, milk yields are depressed in quarters not receiving LPS despite the absence of inflammation in the quarters (Shuster et al., 1991a). Thus, the suppression in milk production is apparently medi-

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ated by systemic effects of intramammary LPS infusion. Intravenous administration of LPS induces many of the systemic responses associated with mastitis, including increases in rectal temperature and serum cortisol (Jackson et al., 1990). On the other hand, there are various indications that LPS is not released from the mammary gland into the general circulation (Dosogne et al., 2002; Hirvonen et al., 1999), but proinflammatory cytokines [e.g. tumor necrosis factor- α (TNF)], Interleukin-1 β , Interleukin-6) produced during APR enter the circulation and cause the systemic effects (Hoebe et al., 2000).

A recent study suggested that TNF plays a vital role in the pathophysiology associated with coliform mastitis and/or APR (Hoebe et al., 2000). Bovine mammary macrophages secrete TNF in response to LPS (Pighetti and Sordillo, 1994), and the serum TNF level increases in coliform mastitis (Hoebe et al., 2000). In fact, the administration of recombinant bovine tumor necrosis factor- α (rbTNF) to calves induced shock and metabolic and hormonal changes in plasma similar to those observed after LPS administration (Kushibiki et al., 2000a). The studies using LPS have been done with lactating cows (Shuster et al., 1991b), but the effects of exogenous TNF treatment on hormonal response, lactational performance and mastitic parameters have not yet been studied. Moreover, little information has been reported on somatotropin (ST), insulin-like growth factor 1 (IGF-1) and thyroid hormone responses to TNF treatment of lactating cows, although these hormones are important regulators of mammary gland function.

The objective of the present study using lactating cows was to investigate some metabolic and hormonal changes and lactational performance during rbTNF-induced APR in order to provide evidence that TNF is an *in vivo* mediator of coliform mastitis-stimulated systemic changes.

MATERIALS AND METHODS

Cows and Feeding

Twelve nonpregnant, lactating Holstein cows (average BW $\pm$ SE, 584.6 $\pm$ 17.1 kg) of 1st to 3rd parity between 10 and 14 wk after parturition were used in this study. They were housed in tie stalls with 12 h of light/d and were milked twice daily at 0600 and 1600 h with a quarter milking machine, and the daily milk yield was recorded. The cows were fed according to Japanese Feeding Standard (1994) recommendations for dairy cows yielding 30 kg/d of milk containing 3.5% fat at 0900 and 1700 h daily. The diet consisted of 41.4% mixed concentrate, 22.0% corn silage, 12.6% alfalfa hay cube, and 24.0% orchardgrass hay (DM basis). Water

and mineral blocks were continuously available. Dry matter intake was recorded for each cow from 3 d prior to the treatment period (d 0 - 6) until 3 d after the challenge. The cows were cared for according to TNAES (1998) based on Consortium (1988).

Experimental Protocol

Twelve Holstein cows were randomly divided into a rbTNF treatment group (n = 6) and a saline treatment group (n = 6) as a control. The rbTNF (2.5 μ g per kg BW per d) or saline (3 ml per head per d) was injected s.c. at 1200 h daily for 7 consecutive d (d 0 - 6). The dose of rbTNF was adjusted according to the BW determined at the beginning of treatment (d 0). The cows were used in a crossover design. The experimental period included a rest period of 3 wks to avoid carryover effects. Highly purified rbTNF was provided by Higeta Shoyu Co., Ltd. (Choshi, Japan). The method of producing this cytokine was described previously (Kushibiki et al., 2000b). Blood samples (4 ml) were collected from the external jugular vein into sodium heparin-containing tubes (Terumo Co., Ltd., Tokyo) at 1130 h daily during the experimental period, and were stored on ice and centrifuged (25 min at 1000 $\times$ g) at 4°C. Plasma was aspirated and stored at -20°C until analyzed for metabolites and hormones. Milk samples from cows were collected twice daily for composition analysis. The milk samples were collected on d 0, 1, 3, 5, and 7. The milk samples consisted of mixtures of evening milk and next-morning milk, and a portion of each sample was stored at -20°C. The rest of the sample was used to prepare skim milk by centrifugation at 1000 $\times$ g and 4°C for 15 min. After the fat layer was discarded, the skim milk was stored at -20°C until analysis for nitrite plus nitrate (NO_x).

On d 6 in the experimental period, we determined the effect of daily administration of rbTNF on somatoliberin (NIAIST) (STRH)-stimulated somatotropin (ST) release (STRH challenge, 0.25 μ g/kg BW). On the challenge day, a catheter was inserted into the left external jugular vein at 1000 h, and was maintained by flushing of heparinized saline solution. The STRH was dissolved in saline at 25.0 μ g/ml. The preparation was injected via the catheter immediately following the rbTNF or saline treatment at 1200 h. Blood samples (4 ml) were collected through the catheter into tubes containing sodium heparin at -15, 0 (just before injection), 5, 10, 15, 20, 30, 45, 60, 90, 120 min after the injection. The samples were kept on ice after collection and centrifuged (25 min at 1000 $\times$ g) at 4°C. The plasma was harvested and stored at -20°C until analyzed for ST.

Analysis of Blood and Milk Components

The concentration of plasma haptoglobin (HP) was determined by a single-immunodiffusion method using a kit (Saikin Kagaku, Sendai, Japan) specific for bovine HP, as described previously (Morimatsu et al., 1992). The limit of detection was 25 $\mu\text{g/ml}$ for HP. Plasma nonesterified fatty acid (NEFA) was measured with a commercially available kit (Wako Pure Chemical Industries, Osaka) using a Hitachi 7070 autoanalyzer (Hitachi Ltd., Tokyo). Cortisol in plasma was determined with a RIA kit from Eiken Chemical (Tokyo). This kit contains a bovine cortisol standard. The plasma concentration of triiodothyronine (T_3) was determined using a RIA kit (Eiken Chemical, Tokyo) previously validated for use in cattle (Rumsey et al., 1990). ST analysis was performed using RIA for bovine ST as described by Johke (1978). Intra- and interassay coefficients of variation were 4.8 and 6.3% for cortisol, 5.2 and 7.7% for T_3 , and 5.5 and 8.7% for ST, respectively. The plasma concentration of IGF-1 in daily samples of each treatment group was determined using a double antibody RIA (Hodate et al., 1990). The intraassay coefficient of variation was 5.1% for IGF-1.

Nitric oxide (NO) production was evaluated by measuring its more stable metabolite, NO_x. The concentrations of NO_x in plasma and milk were the sum of the nitrite (NO₂) and nitrate (NO₃) levels. Immediately prior to the determination of NO_x in milk, the skim milk samples were centrifuged twice for 15 min at 10,000 $\times$ g at 4°C, and the whey fraction between the supernatant (fat layer) and the infranatant (precipitates) was removed. To measure NO_x, NO₃ was converted to NO₂ by the addition of NO₃ reductase, and NO₂ was then measured at 540 nm by the Griess reaction (Blum et al., 2000).

Milk fat and protein were measured with an infrared milk analyzer.

Statistical Analysis

Data were analyzed using a repeated measurement design with the GLM procedure of SAS (1988). The model was $Y_{ijk} = \mu + A_i + B_j + C_k + (AC)_{ik} + e_{ijk}$, where Y_{ijk} = observed value, μ = overall mean, A_i = effect of treatment, B_j = effect of individual cows, C_k = effect of sampling day or time, $(AC)_{ik}$ = interaction of treatment and sampling day or time, and e_{ijk} = random residual error.

All data are presented as mean $\pm$ SE and $P < 0.05$ was considered significant.

Figure 1. Plasma concentrations of haptoglobin (HP) in cows given s.c. injections of saline (control, 3 mL/head) or recombinant bovine tumor necrosis factor- α (rbTNF, 2.5 $\mu\text{g/kg}$) once daily for 7 d. Blood samples were collected from the jugular vein at 1130 h daily during the period from d 0 until 7. The saline or rbTNF was administered at 1200 h daily for 7 d (d 0 – 6). Each point represents the mean $\pm$ SE; 12 cows per treatment. * $P < 0.05$ compared to control.

RESULTS

Feed Intake, Blood Metabolites and Hormones

The rbTNF treatment produced some local inflammatory reaction (heat, swelling) around the place of s.c. injection during the period between d 1 and 9.

During the treatment (d 0 – 6) and posttreatment (d 7 – 9) periods, DM intake for cows treated with rbTNF was depressed ($P < 0.05$) 34.3 and 20.0% when compared to control cows (data not shown).

The concentration of HP in the cows treated with rbTNF was already elevated ($P < 0.05$) at d 1 and remained significantly higher than that in the control cows throughout the experimental period (Figure 1).

Daily rbTNF administration increased the mean NEFA concentration ($P < 0.05$) from d 3 to 7 compared with the level in control cows (Figure 2).

In the cows treated with rbTNF, the plasma concentration of cortisol was increased during the period between d 2 and 7 compared with that in the control cows (Figure 3, upper panel). The plasma T_3 concentration in the rbTNF-injected cows was decreased ($P < 0.05$) at d 1 and remained low throughout the experimental period (Figure 3, lower panel).

In the rbTNF group, the plasma concentration of ST was increased throughout the experimental period compared with that of the control group (Figure 4, upper panel). On d 2 – 7, there was a significant difference

Figure 2. Plasma concentrations of NEFA in cows given s.c. injections of saline (control, 3 mL/head) or rbTNF (2.5 μg/kg) once daily for 7 d. See Figure 1 for key.

($P < .05$) between the plasma concentrations of ST in the rbTNF and control groups.

The concentration of plasma IGF-1 in the cows treated with rbTNF tended to be reduced at d 1 and was significantly lower ($P < .05$) than in the control cows throughout the period from d 3 to 7 (Figure 4, lower panel).

STRH Challenge

The ST concentrations in both groups were elevated at 5 min after the STRH administration, but plasma ST was significantly lower ($P < 0.05$) in the rbTNF-treated cows than in the control cows from 15 to 120 min after the STRH administration (Figure 5).

Concentrations of NO_x in Plasma and Milk

Plasma NO_x concentration in the cows treated with rbTNF was increased ($P < 0.05$) at 1 d and remained elevated level until 7 d compared with that for the cows treated with saline (Figure 6, upper panel). Similarly, milk NO_x concentration in the rbTNF-injected cows was higher ($P < 0.05$) than that for the control cows during from 1 to 7 d (Figure 6, lower panel).

Milk Yields and Compositions

Treatment with rbTNF decreased the milk yield ($P < 0.05$) during the period between d 1 and 8 (Figure 7). The milk fat percentage was elevated ($P < 0.05$) at d 1 following rbTNF treatment and remained higher until

Figure 3. Plasma concentrations of cortisol (upper panel) and triiodothyronine (T₃, lower panel) in cows given s.c. injections of saline (control, 3 mL/head) or rbTNF (2.5 μg/kg) once daily for 7 d. See Figure 1 for key.

d 7 in comparison with that in the saline-treated cows (Figure 8, upper panel). The protein content in milk was lower ($P < 0.05$) in the rbTNF-treated cows than in the control cows from d 5 to 7 (Figure 8, lower panel).

DISCUSSION

Daily treatment with rbTNF induced increases in plasma HP and NEFA levels, and altered hormone concentrations in lactating cows. In addition, rbTNF administration caused an elevation of the NO_x level in plasma and milk, and influenced lactational performance.

HP is produced mainly in the liver in response to stimulation by proinflammatory cytokines such as TNF,

Figure 4. Plasma concentrations of ST (upper panel) and IGF-1 (lower panel) in cows given s.c. injections of saline (control, 3 mL/head) or rbTNF (2.5 μ g/kg) once daily for 7 d. See Figure 1 for key.

which are produced primarily by macrophages and monocytes (Baumann and Gauldie, 1994). This acute phase protein fulfills a number of nonspecific defense functions during the APR (Baumann and Gauldie, 1994). In cattle, plasma HP is a major contributor to the APR (Alsemgeest et al., 1994). We previously demonstrated that a single injection of rbTNF in calves resulted in a dose-dependent increase of the plasma HP level (Kushibiki et al., 2000a).

It has been reported that TNF is responsible for the hyperlipidemia that occurs in association with inflammation or infection (Feingold and Grunfeld, 1987). In mice (Green et al., 1994), TNF causes a delipidation of fat cells and a decrease of the adipose tissue mass.

Figure 5. Time course of the level of plasma ST after an i.v. injection of STRH (0.25 μ g/kg) on d 6 in cows treated with saline (control) or rbTNF. The treatments consisted of rbTNF (2.5 μ g/kg/d s.c.) and saline (3 mL/d s.c.) given by daily injection for 7 d (d 0 - 6). * P < 0.05 compared to control.

Hepatic lipid synthesis is also stimulated by TNF administration (Feingold and Grunfeld, 1987). Previously, we demonstrated that a single i.v. administration of rbTNF to heifers resulted in an increase in the plasma NEFA concentration (Kushibiki et al., 2000b). The increased NEFA level observed here in the lactating cows treated with rbTNF was similar to that observed previously in vivo studies. Moreover, when lactating cows are in negative energy balance, lipid reserves are mobilized and oxidation of NEFA is increased, thereby preserving the limited supplies of other key nutrients such as glucose and amino acids (Peel and Bauman, 1987). In the present study, it is possible that the reduction in DM intake during rbTNF treatment resulted in an increase in the circulating NEFA concentration that was highly correlated with energy balance in the lactating cows.

Intramammary or i.v. administration of endotoxin to lactating cows induces many systemic responses associated with mastitis, including an increase in the plasma cortisol level (Jackson et al., 1990). This response results from an endotoxin-induced release of cytokines, e.g., TNF, from peripheral blood monocytes and liver Kupffer cells (Lohuis et al., 1988a). TNF stimulates the hypothalamic-pituitary-adrenal axis in rats, mainly through an effect on the hypothalamus (Bernardini et al., 1990). In the present study, exogenous rbTNF treatment elicited a pronounced elevation of the plasma cortisol concentration in lactating cows.

Figure 6. Concentrations of nitrite plus nitrate (NOx) in plasma (upper panel) and milk (lower panel) of cows treated with saline (control, 3 mL/head) or rbTNF (2.5 μg/kg) for 7 d (d 0–6). See Figure 1 for key.

Thyroid hormones are also important for maintaining lactation, and stimulate the basic metabolic rate via enhancing the metabolism of carbohydrates, lipids, and proteins (Nixon et al., 1988). T_3 is the most metabolically active thyroid hormone (Refsal et al., 1984). The concentration of plasma T_3 was related positively with the basic metabolic rate of lactating cows (Refsal et al., 1984). On the other hand, TNF inhibits the synthesis and secretion of T_3 and mediates alterations in thyroid hormone metabolism in rats (Pang et al., 1989). In our study, rbTNF treatment induced a decrease of the plasma T_3 concentration compared with that in the control cows, suggesting suppression of the basic metabolic

Figure 7. Milk yield of cows treated with saline (control, 3 mL/head) or rbTNF (2.5 μg/kg) during the pre-treatment (d –1), treatment (d 0–6), and posttreatment (d 7–10) periods. The saline or rbTNF was administered at 1200 h daily for 7 d (d 0–6). Values are means for 12 cows with SE represented by vertical bars. * $P < 0.05$ compared with control.

rate and the synthesis and secretion of T_3 by rbTNF in the lactating cows.

In the present study, the basal concentration of ST in plasma was increased by rbTNF administration. In contrast, daily rbTNF treatment induced a decrease in the plasma concentration of IGF-1 in the lactating cows. These findings are in agreement with previous reports that investigated the effect of negative energy balance, restricted feed or mastitis on the plasma ST and IGF-1 concentrations in lactating cows (Burvenich et al., 1999; Gluckman et al., 1987). Furthermore, a recent study suggested that i.v. administration of endotoxin decreases the plasma concentration of IGF-1 in steers, and that the effect is not totally explained by the changes in voluntary feed intake that accompany endotoxin administration (Elsasser et al., 1995). In rats, TNF inhibited the expression of the IGF-1 and IGF-1 receptor genes in liver, in association with a reduction in the concentration of IGF-1 in the plasma (Char et al., 1995). On the other hand, the plasma ST response to exogenous STRH was lower in the rbTNF-treated cows than in the control cows. This result is in agreement with the results obtained in our previous studies using heifers (Kushibiki et al., 2000b) and steers (Kushibiki et al., 2001). A recent study indicated that TNF receptors are found in the bovine pituitary gland (Elsasser et al., 1991). In addition, TNF inhibits STRH-stimulated ST release in cultured bovine pituitary cells (Elsasser et al., 1991).

Figure 8. Percentages of fat (upper panel) and protein (lower panel) in milk of cows treated with saline (control, 3 mL/head) or rbTNF (2.5 μ g/kg) for 7 d (0–6 d). Values are means for 12 cows with SE represented by vertical bars. * P < 0.05 compared with control.

NO acts as a mediator of many of the pathological consequences of endotoxin or infection, and is generated in response to various cytokines, especially TNF (Cunha et al., 1994). In vivo studies using lactating cows have suggested that NO has important metabolic and endocrine effects (Blum et al., 2000; Hirvonen et al., 1999). The plasma NO_x concentration was also increased by intramammary challenge with *E. coli* (Blum et al., 2000; Hoeben et al., 2000). These observations may indicate that there is a close relationship between TNF and NO production during coliform mastitis in lactating cows (Blum et al., 2000; Hoeben et al., 2000). Our data provide the first evidence that exogenous

rbTNF treatment elevated NO_x production in the plasma and milk of lactating cows.

In studies using the mastitis model to investigate the pathophysiological causes of reduced lactational performance during mastitis, milk yields in early lactation were depressed in quarters not receiving *E. coli* despite the absence of inflammation in these quarters (Heyneman et al., 1990; Hoeben et al., 2000). In contrast, milk production in early lactation decreased rapidly, but was less pronounced, after intramammary infusion of endotoxin (Hoeben et al., 2000). The milk production losses in the untreated quarters were negligible in endotoxin mastitis (Hoeben et al., 2000). Thus, the suppression of milk production was apparently mediated in part by systemic effects of intramammary *E. coli* infusion, and may be caused by changes in the concentrations of stimulatory or inhibitory hormones (e.g., ST, T₃, and cortisol) (Hoeben et al., 2000; Lohuis et al., 1988b; Shuster et al., 1991c). The stage in lactation is an important epidemiologic factor of the relationship between milk production loss and mastitis (Bartlett and Van Wijk, 1991). Several studies have reported on the differences in the milk production patterns following clinical mastitis between the stage of early and middle to late lactation (Lescourret and Coulon, 1994; Lucey and Rowlands, 1984). Most cases of acute or peracute coliform mastitis occur from calving to peak lactation (Lohuis et al., 1990). Data from the present study indicated that rbTNF induced systemic responses associated with mastitis in peak lactation, including a decrease in milk production, an increase of fat content and a decrease of protein content in milk. These changes in milk composition were similar to those observed following i.v. endotoxin treatment (Shuster et al., 1991b). Also, there was a significant increase in fat concentration in milk from the uninfected quarters after intramammary injection of *E. coli* (Massart-Leen et al., 1994). It is likely that the increased milk fat percentage was mediated by the rbTNF-induced catabolic state of the host (e.g., hepatic lipogenesis and decreased adipose tissue mass) and the suppression of milk production. On the other hand, an in vitro study showed that TNF inhibited the secretion of casein from bovine mammary cells (Hurley et al., 1994). Watanabe et al. (2000) demonstrated in vivo that the concentrations of casein, β -lactoglobulin, and α -lactalbumin in milk were significantly decreased following intramammary rbTNF infusion.

In conclusion, this study showed that exogenous rbTNF injection induced many of the systemic responses associated with coliform mastitis in the plasma and milk of lactating cows. These findings support and confirm the hypothesis (Dosogne et al., 2002; Hoeben et al., 2000) that TNF, rather than LPS, is absorbed from the mammary gland and released into the circula-

tion, and that TNF is responsible for the systemic changes observed during coliform mastitis.

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