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Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows

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ABSTRACT

Metabolic adaptations during negative energy and nutrient balance in dairy cows are thought to cause impaired immune function and hence increased risk of infectious diseases, including mastitis. Characteristic adaptations mostly occurring in early lactation are an elevation of plasma ketone bodies and free fatty acids (nonesterified fatty acids, NEFA) and diminished glucose concentration. The aim of this study was to investigate effects of elevated plasma β-hydroxybutyrate (BHBA) at simultaneously even or positive energy balance and thus normal plasma NEFA and glucose on factors related to the immune system in liver and mammary gland of dairy cows. In addition, we investigated the effect of elevated plasma BHBA and intramammary lipopolysaccharide (LPS) challenge on the mammary immune response. Thirteen dairy cows were infused either with BHBA (HyperB, n = 5) to induce hyperketonemia (1.7 mmol/L) or with a 0.9% saline solution (NaCl, n = 8) for 56 h. Two udder quarters were injected with 200 μg of LPS after 48 h of infusion. Rectal temperature (RT) and somatic cell counts (SCC) were measured before, at 48 h after the start of infusions, and hourly during the LPS challenge. The mRNA abundance of factors related to the immune system was measured in hepatic and mammary tissue biopsies 1 wk before and 48 h after the start of the infusion, and additionally in mammary tissue at 56 h of infusion (8 h after LPS administration). At 48 h of infusion in HyperB, the mRNA abundance of serum amyloid A (SAA) in the mammary gland was increased and that of haptoglobin (Hp) tended to be increased. Rectal temperature, SCC, and mRNA abundance of candidate genes in the liver were not affected by the BHBA infusion until 48 h. During the following LPS challenge, RT and SCC increased in both groups. However, SCC increased less in HyperB than in NaCl. Quarters infused with LPS showed a more pronounced increase of mRNA abundance of IL-8 and IL-10 in HyperB than in NaCl. The results demonstrate that an increase of plasma BHBA upregulates acute phase proteins in the mammary gland. In response to intramammary LPS challenge, elevated BHBA diminishes the influx of leukocytes from blood into milk, perhaps by via modified cytokine synthesis. Results indicate that increased ketone body plasma concentrations may play a crucial role in the higher mastitis susceptibility in early lactation.
Key words: hyperketonemia, immune response, lipopolysaccharide, dairy cow

INTRODUCTION

For several decades, dairy cows have been selected for high milk production. With the increase in milk production, the incidence of infectious diseases has increased (Syväjärvi et al., 1986; Simianer et al., 1991; Uribe et al., 1995). Mainly during the first weeks of lactation, cows experience immunosuppression and have a high susceptibility to infectious diseases (Smith et al., 1985; Hogan et al., 1989; Goff, 2006), which is thought to be due to the metabolic adaptations to negative energy balance (**NEB**; Suriyasathaporn et al., 2000; van Dorland et al., 2009). Low plasma glucose concentrations and elevated plasma NEFA and ketone body concentrations, specifically BHBA, are the characteristic changes during NEB (Bobe et al., 2004; van Dorland et al., 2009; Gross et al., 2011). Increased plasma BHBA concentrations above 1,200 μmol/L are considered indicative of a metabolic disorder (Ospina et al., 2010) and defined as subclinical ketosis (Duffield et al., 2009).

The occurrence of mastitis is determined by several pathogen, animal, and environmental factors (Burvenich et al., 2007). Among the animal factors, high plasma BHBA concentration has a negative influence on the susceptibility of mastitis and course of disease (Heyneman et al., 1990; Oltenacu and Ekesbo, 1994; Van Werven et al., 1997). In vitro, growth of bacteria

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obtained from LPS-challenged quarters in hyperketotic and normal dairy cows showed that high plasma BHBA concentrations had a high positive correlation with the severity of mastitis induced by *Escherichia coli* (Kremer et al., 1993). In addition, elevation of plasma BHBA concentration decreased in vitro chemotaxis and microbial killing in human neutrophils (McMurray et al., 1990) and respiratory burst activity of bovine neutrophils (Hoeben et al., 1997). Cytokine production was reduced after bacterial infection in ketotic dairy cows (Filar et al., 1992; Kandefer-Szerszen et al., 1992). However, elevated BHBA occurs mostly concomitantly with other metabolic and endocrine changes (Kessel et al., 2008; Gross et al., 2011), and the immunosuppressive effect cannot be exclusively ascribed to the ketone bodies. Results of exclusive BHBA effects on the immune system are available from in vitro studies. Thus, in the presence of BHBA, a decreased phagocytotic activity of milk polymorphonuclear leukocytes was demonstrated (Kluciński et al., 1988), as well as reduced chemotactic capacity of bovine blood leukocytes (Suriyasathaporn et al., 1999). The role of BHBA in immunosuppression in vivo is not fully understood (Burton et al., 2005). Information about the effects of hyperketonemia isolated from other metabolic changes on the immune response and SCC in hyperketotic cows is rare, and the effects of long-term hyperketonemia have not been investigated in dairy cows. The objective of this work was to investigate effects of a 48-h elevation of plasma BHBA, through BHBA infusion, on parameters related to the immune system in liver and mammary gland, and to study the mammary immune response to LPS challenge during elevated BHBA for additional 8 h.

MATERIALS AND METHODS

Animals and Management

All procedures involving animals followed the Swiss Animal Protection Law and were approved by the Federal Veterinary Office in Switzerland. Thirteen nonpregnant Holstein dairy cows in diestrus with a parity of 3.5 ± 0.10, at 28 ± 0.3 (mean ± SD) wk in milk were selected. Cows in mid lactation were chosen as the best possible model for investigation of the specific effect of BHBA infusion without the comprehensive endocrine and metabolic changes that occur during the period of highest milk production in early lactation. The cows were free of mastitis and other infectious diseases, which was confirmed by a routine blood glutaraldehyde coagulation test (Sandholm, 1976), and by measuring milk SCC (DeLaval cell counter DCC, DeLaval International AB, Tumba, Sweden), which had to be $<150 \times 10^3$ cells/mL in all 4 quarters at the start of the experiment. The validity and repeatability of the DeLaval cell counter was previously shown (Sarikaya and Bruckmaier, 2006).

Animals were moved to the experimental tiestall for adaptation to housing and feeding conditions 2 wk before the start of the experiment. Both groups were fed ad libitum with hay (DM content, 890 g/kg of fresh matter; on DM basis, consisting of 153 g of CP/kg, 235.0 g of crude fiber/kg, and 5.7 MJ of NE_L/kg). In addition, cows received a protein- and energy-rich concentrate (23.5% barley, 14.0% oats, 20.0% wheat bran, 17.0% soybean expeller, 15.0% linseed meal, 0.6% salt, 2.2% carbonate of lime, 0.4% calf rearing feed premix, 4.0% molasses, and 3.0% bypass fat, DM content, 881 g/kg of fresh matter; on DM basis, consisting of 217 g of CP/kg, 73.9 g of crude fiber/kg, and 7.6 MJ of NE_L/kg) twice daily according to individual milk production. In addition, minerals (50 g/cow) were supplied once a day. Animals had access to fresh water ad libitum. Milking was performed twice daily at 0530 and 1600 h.

Experimental Design and Treatments

Cows were randomly allocated to 1 of 2 infusion treatment groups, as described earlier (Zarrin et al., 2013). In brief, the treatments involved an intravenous Na-DL-β-OH-butyrate infusion to obtain a plasma BHBA concentration between 1.5 and 2.0 mmol/L (**HyperB**, n = 5), comparable to those in spontaneous hyperketonemia, and a saline (0.9%) infusion (NaCl, 20 mL/h, n = 8) as the control group. One day before the infusions started, cows were fitted with indwelling intravenous catheters (16-gauge; Cavafix Certo Splittocan, B. Braun Melsung AG, Melsungen, Germany) with a length of 32 cm in both jugular veins. The infusions started at 0900 h on d 1 and continued until 1700 h 2 d later. After 48 h of infusions, each of 2 udder quarters was injected with 200 μg of LPS *Escherichia coli* serotype 026:B6 (#L8274; Sigma-Aldrich, St. Louis, MO) as LPS quarters, and 0.9% NaCl solution were injected in 2 udder quarters as control. Details were described by Vernay et al. (2012).

Data Collection and Sampling

Blood Samples. Baseline blood samples at 1 wk and at 2 h before the start of the infusion were taken between milking and feeding at 0730 h. Sampling was continued at least hourly during the 56-h infusion period. During the experimental infusions, blood samples were obtained from the contralateral jugular catheter, which was not used for infusion, using tubes containing tri-potassium-EDTA. Plasma was separated by centrifugation at 3,000 × *g* for 20 min, and stored at −20°C

until analyses. Every 15 min during the first 2 h and every hour during the entire infusion period, additional small (1 mL) blood samples were analyzed immediately for adjustment of BHBA infusion rates to achieve and maintain the intended plasma concentration.

Rectal Temperature. Rectal temperature was measured during the adaptation period, immediately before the start of infusions, 2 d after the start of infusion, and hourly during the LPS challenge (for 8 h).

Milk Samples. Foremilk samples (~50 mL) were obtained before morning milking, after teat cleaning from both front quarters (one challenged with LPS and one control) during the adaptation period, immediately before the start of infusion, 2 d after the start of infusion, and hourly during the LPS challenge (8 h). Milk SCC was measured with a DeLaval cell counter (DeLaval International AB) immediately after sampling.

Udder Biopsies. One week before the start of infusions, 48 h after the start of the infusions (before LPS challenge), and at the end of experiment (56 h), udder tissue biopsies were taken from the 2 rear quarters. Before the biopsies, cows were sedated by an intravenous injection of 16 μg/kg of BW of xylazine (Xylazin Streuli ad us. Vet.; G. Streuli & Co. AG, Uznach, Switzerland). Udder tissue (30 to 60 mg) was obtained under local anesthesia with 10 mL of lidocaine 2% (Streuli Pharma AG, Uznach, Switzerland), using a 12-gauge, 10-cm biopsy needle (Bard Magnum Core Tissue Biopsy Needle, Türkenfeld, Germany). The samples were placed immediately into RNA stabilization reagent (RNAlater, Ambion/Applied Biosystems, Austin, TX), kept at 4°C for 24 h, and stored at –80°C until RNA extraction.

Liver Biopsies. Liver tissue (60 to 100 mg) was taken 1 wk before the infusions and at 48 h after the start of infusions, under local anesthesia with 10 mL of lidocaine 2% (Streuli Pharma AG) as described previously (Zarrin et al., 2013). The samples were kept at 4°C for 24 h in RNA stabilization reagent (RNAlater, Ambion/Applied Biosystems) and stored at –80°C until RNA extraction.

Laboratory Procedures

Udder and Liver Tissue. Total RNA was extracted from udder and liver tissues with peq GOLD TriFast (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. Quantity and purity of RNA were measured by using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) at 260 and 280 nm absorbance waves. Complementary (c)DNA was made by reverse transcription of 1 μg of total RNA with Moloney murine leukemia virus Reverse Transcriptase RNAase H Minus, Point Mutant (MMLV-RT; Promega Corp., Madison, WI) and random hexamer primers (Invitrogen, Leek, the Netherlands). The mRNA abundances of housekeeping genes (*GAPDH* and ubiquitin) and target genes related to immune response were measured by real-time quantitative PCR in a Rotor-Gene 6000 rotary analyzer (Corbett Research, Sydney, Australia) and software version 1.7.75, using a master mix consisting of 0.8 μL of PCR water, 1.0 μL of forward primer (5 pmol), 1.0 μL of reverse primer (5 pmol), and 5.2 μL of 2× SensiMix plus SYBR-Green. The cycle threshold (**CT**) values obtained for candidate genes were adjusted according the mean of the housekeeping genes CT according to the following equation: ΔCT = CT (arithmetic mean of housekeeping genes) – CT (target gene). Differences in mRNA abundance during 48-h BHBA infusion in udder and liver were calculated according to the following equation: ΔΔCT = ΔCT [d 2 (48 h)] – ΔCT [d 0 (0 h)]. Differences in mRNA abundance during LPS challenge were calculated for LPS and control quarters separately according to this equation: ΔΔCT = ΔCT [d 3 (8 h)] – ΔCT [d 3 (0 h)], as described by Vernay et al. (2012). The primer sequences for housekeeping and candidate genes were used according to previous publications: GAPDH, IL-8, serum amyloid A (**SAA**), and tumor necrosis factor-α (**TNFα**) (Wellnitz et al., 2006), ubiquitin, lactoferrin, and inducible nitric oxide synthase (**iNOS**) (Schmitz et al., 2004), nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**) (Bruckmaier and Meyer, 2005), Haptoglobin (**Hp**) (Hiss et al., 2004), and IL-1β (Griesbeck-Zilch et al., 2008). The selected and measured genes, primer sequences, and the PCR conditions are shown in Table 1.

Statistical Analysis

Data are presented as means ± SEM, and differences were considered significant if $P < 0.05$ and as a tendency if $P < 0.10$.

Differences between before and after 48-h BHBA infusion and before and after LPS challenge (delta) were calculated for all recorded parameters. Somatic cell counts were analyzed and are shown on the logarithmic scale ($\log_{10}$) to ensure normal distribution of data. Area under the curve was calculated for RT and SCC during the LPS challenge (8 h). Mammary mRNA abundance data were analyzed by using the general linear models (GLM) procedure of SAS (release 9.2; SAS Institute Inc., Cary, NC), including treatment (BHBA or NaCl) as a fixed effect. Differences between means were evaluated by the Tukey test. In addition, means of delta values obtained within each treatment were tested for differences from zero. Differences in SCC and RT be-

Table 1. Primer information (for = forward, rev = reverse), annealing temperature, and the PCR product length

Gene[1]	Primer sequence 5′ to 3′		GenBank accession no.	Annealing temperature (°C)	Length (bp)
GAPDH	for	GTC TTC ACT ACC ATG GAG AAG G	NM001034034	60	197
	rev	TCA TGG ATG ACC TTG GCC AG			
Ubiquitin	for	AGA TCC AGG ATA AGG AAG GCA T	NM174133	62	198
	rev	GCT CCA CCT CCA GGG TGA T			
IL-1β	for	AGT GCC TAC GCA CAT GTC TTC	M37211	60	114
	rev	TGC GTC ACA CAG AAA CTC GTC			
IL-6	for	CTTCACAAGCGCCTTCACTC	NM_173923.2	62	132
	rev	GTCAGAAGTAGTCTGCCTGG			
IL-8	for	ATG ACT TCC AAG CTG GCT GTT G	AF232704	60	149
	rev	TTG ATA AAT TTG GGG TGG AAA G			
IL-10	for	CCT GGA AGA GGT GAT GCC AC	EU276074	60	133
	rev	GTT TTC GCA GGG CAG AAA GCG			
iNOS	for	ACC TAC CAG CTG ACG GGA GAT	U14640	62	316
	rev	TGG CAG GGT CCC CTC TGA TG			
TNFα	for	CCA CGT TGT AGC CGA CAT C	NM173966	60	155
	rev	CCC TGA AGA GGA CCT GTG AG			
Lactoferrin	for	GGC CTT TGC CTT GGA ATG TAT	L08604	62	338
	rev	ATT TAG CCA CAG CTC CCT GGA G			
NF-κB	for	GCT GGA CCC AAG GAC ATG	NM003998	56	235
	rev	TGG TCT GCT GCA GAG CTG			
Hp	for	GTCTCCCAGCATAACCTCATCTC	AJ271156	55	174
	rev	AACCACCTTCTCCACCTCTACAA			
SAA	for	CCT GGG CTG CTA AAG TGA TC	AF540564	57	184
	rev	TAC TTG TCA GGC AGG CCA G			

[1]GAPDH = glyceraldehyde 3-phosphate dehydrogenase; iNOS = inducible nitric oxide synthase; TNFα = tumor necrosis factor-α; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; Hp = haptoglobin; SAA = serum amyloid A.

tween treatments within each time point and between time points within treatments were evaluated by using the ANOVA mixed model procedure of SAS, including treatment (BHBA or NaCl), time, and their interactions as fixed effects and cows as repeated subject.

RESULTS

As reported previously, the mean infusion rate of BHBA during 48 h was 8.1 ± 0.3 μmol/kg per minute, and plasma BHBA concentration was maintained at 1.7 ± 0.1 mmol/L in the HyperB group (Zarrin et al., 2013). In the control group, plasma BHBA concentration was 0.6 ± 0.1 mmol/L. In response to LPS administration at 48 h of infusion, plasma BHBA started to decrease ($P < 0.01$) and the infusion rate had to be increased ($P < 0.001$) to 11.1 ± 1.0 μmol/kg per minute to maintain hyperketonemia (Figure 1). Despite the increased BHBA infusion rate after LPS administration, mean plasma BHBA concentration after LPS challenge (8 h) was not fully maintained and was 1.4 ± 0.1 mmol/L in HyperB. In the control group, plasma BHBA also decreased ($P < 0.01$) after LPS administration (0.4 ± 0.1 mmol/L).

SCC and Rectal Temperature

β-Hydroxybutyrate infusion did not affect RT or milk SCC during 48 h of BHBA infusion (before LPS challenge; data not shown). The subsequent intramammary LPS challenge caused a significant increase in SCC in LPS-challenged quarters compared with control quarters in both groups within 3 h after LPS administration (Figure 2). The increase in SCC was lower in the HyperB group than in the control group ($P < 0.01$). In response to LPS challenge, RT increased similarly in both groups from 38.6 ± 0.0°C and 38.1 ± 0.2°C to a maximum of 40.5 ± 0.2°C and 40.8 ± 0.1°C, reached at 6 and 5 h after LPS administration, in HyperB and NaCl, respectively.

Changes of mRNA Abundance During BHBA infusion in Liver and Mammary Tissue Before LPS Challenge

Changes in mRNA abundance of candidate genes related to immune response in liver and mammary tissue are shown in Table 2. During 48 h of infusion, the mRNA abundance of SAA and Hp in the liver increased similarly in both groups.

In mammary tissue, the mRNA abundance of SAA increased during 48 h of infusion in HyperB ($P < 0.01$) but not in the control group. Haptoglobin mRNA abundance tended to increase in HyperB compared with the control group ($P = 0.07$). The mRNA abundance of the other measured candidate genes in the mammary gland did not change during the first 48 h of BHBA and NaCl infusion.

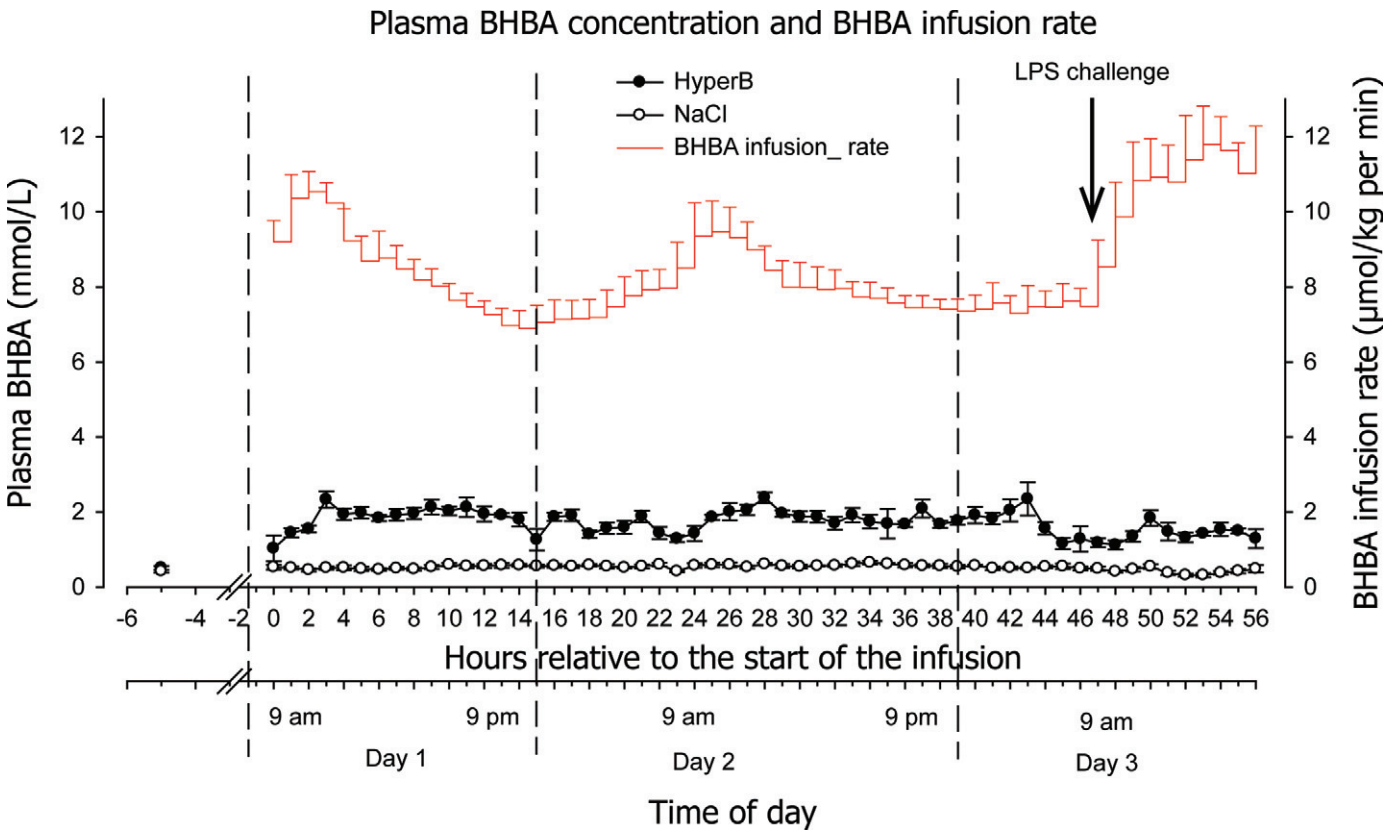

Figure 1. Plasma BHBA and BHBA infusion rate during a 56-h infusion in cows infused with BHBA (HyperB) and cows infused with NaCl as control group (NaCl). At 48 h of infusion 2 quarters of both treatment groups were challenged by intramammary LPS. Values represent means ± SEM. Color version available in the online PDF.

Changes in mRNA Abundance in Mammary Tissue in Response to LPS Challenge

Changes in mRNA abundance of candidate genes in mammary tissue related to the immune response to LPS are shown on Table 3. Intramammary LPS challenge caused an increased mRNA abundance of IL-1β, IL-6, IL-8, IL-10, TNFα, iNOS, NF-κB, Hp, and SAA in the LPS-challenged quarters in both treatment groups. The mRNA abundance of IL-6, IL-10, and Hp also increased in the control quarters in both treatment groups. In the HyperB group, the increase of mammary mRNA abundance in quarters with LPS administration was more pronounced than in the control group for IL-8 ($P < 0.01$) and IL-10 ($P < 0.05$), respectively.

DISCUSSION

To the best of our knowledge, the present experiment is the first study to investigate the effects of long-term hyperketonemia through BHBA infusion on SCC and immune response in dairy cows that were stimulated with intramammary *E. coli* LPS.

Unchanged SCC in milk during 48 h of increased BHBA concentrations in blood in the present study was in contrast to a previous study (Duffield et al., 1998) that reported an association between hyperketonemia and increased SCC after parturition in dairy cows. However, the BHBA infusion represented an additional energy source during the already positive energy balance in the present study compared with the NEB that was reported in the mentioned study. In addition, in the present study, the induced hyperketonemia by BHBA infusion was completely different from spontaneous hyperketonemia during NEB that is accompanied by low plasma glucose concentration and high plasma NEFA concentration after parturition (Kessel et al., 2008; van Dorland et al., 2009; Gross et al., 2011).

Consistent with previous studies, LPS challenge caused an increase in body temperature (Schmitz et al., 2004; Vernay et al., 2012) and milk SCC (Wellnitz et al., 2011; Vernay et al., 2012). The induced increase of RT confirmed that LPS challenge caused not only a local activation of the immune response but also had a systemic effect (Dinarello, 1991). An increase in SCC is accepted as an indicator of inflammatory responses in

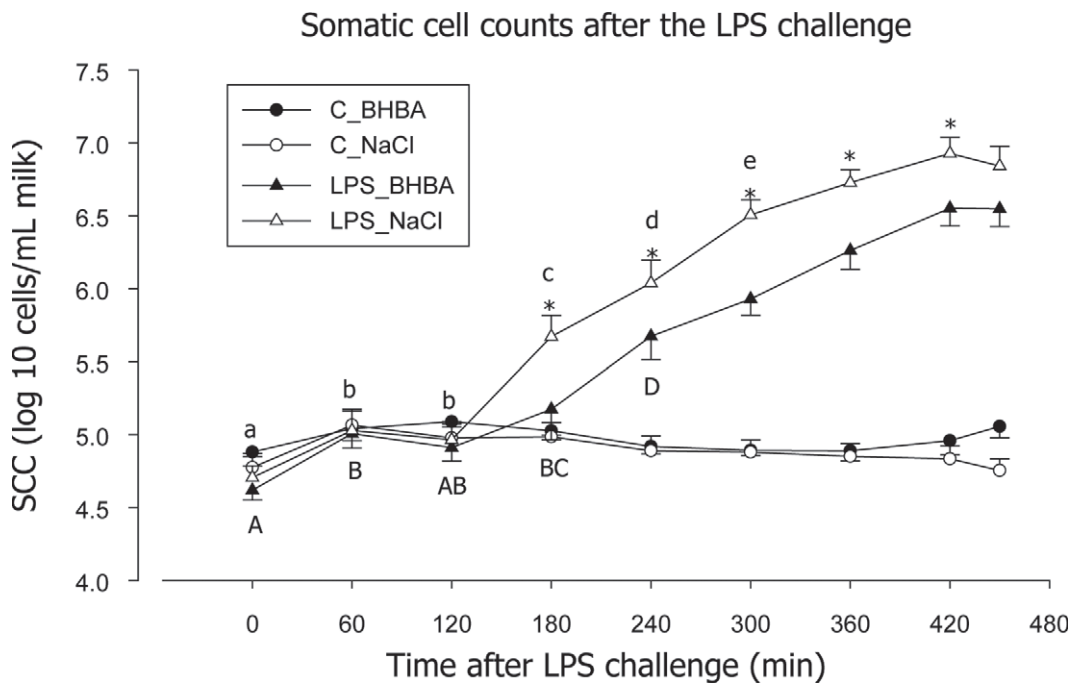

Figure 2. Somatic cell counts (in $\log_{10}$ scale) in milk after the LPS challenge. Data are means ± SEM in quarters that were challenged with intramammary *Escherichia coli* LPS (LPS_BHBA and LPS_NaCl) and quarters that received NaCl (C_BHBA and C_NaCl), in cows that were infused with BHBA and cows that were infused with NaCl as control group, respectively. $^{A–D, a–e}$LPS quarters without a common letter are significantly different ($P < 0.05$) from baseline (time 0 of LPS challenge) in HyperB and control group, respectively. *Differences between SCC in LPS quarters ($P < 0.05$) in HyperB and control group.

the mammary gland (Pfaffl et al., 2003; Schukken et al., 2003; Wellnitz et al., 2011). Interestingly, the increase in milk somatic cells was less pronounced in LPS quarters in HyperB than in the control group. This is supported by earlier in vitro studies that illustrated that the elevation of BHBA concentration decreased neutrophil chemotactic response in bovine milk leukocytes in vitro (Kluciński et al., 1988; Cerone et al., 2007). Therefore, it can be assumed that the diminished increase in SCC in the present study in the HyperB group compared with the control group is related to the negative effects of plasma BHBA concentration on neutrophil recruitment.

Increased hepatic mRNA abundance of SAA and Hp in both treatment groups during 48-h BHBA infusion indicates that the changes are induced by the experimental procedures independent of the applied treatment. It is possible that an increase of SAA and Hp mRNA abundance in the liver is related to experimental stress in both treatment groups. The long-term BHBA infusion did not specifically affect hepatic mRNA abundance of these candidate genes. This finding is in agreement with a previous study illustrating that ketosis does not affect hepatic Hp and SAA mRNA abundance in dairy cows (Loor et al., 2007), and is in contrast to a study that documented increased serum Hp concentration during ketosis (Stengärde et al., 2008). In contrast to that in the liver, SAA mRNA abundance in mammary tissue was increased by long-term BHBA infusion, and Hp tended to increase in HyperB compared with NaCl during 48-h BHBA infusion in the present study. The different effects of BHBA infusion on SAA and Hp mRNA abundance in the mammary tissue is most likely related to a higher sensitivity of SAA than Hp (Gruys et al., 1993; Alsemgeest et al., 1994; Werling et al., 1996). The different effect of high plasma BHBA concentration (48 h) on acute phase protein mRNA abundance in the liver and in the udder might be related to the role of the liver in the systemic immune response, whereas the udder tissue acts mainly locally within the organ.

Increased SAA and Hp mRNA abundance in mammary tissue in response to LPS challenge confirms previous studies that illustrated increased synthesis of SAA (Wellnitz and Kerr, 2004; Bruckmaier and Meyer, 2005; Vernay et al., 2012) and Hp (Hiss et al., 2004) in mammary tissue in response to LPS. Increased udder mRNA abundance of IL-1β, IL-6, iNOS, and TNFα in quarters that were stimulated by intramammary LPS challenge in both groups is related to immune system activation and was documented in previous studies (Hiss et al., 2004; Bruckmaier and Meyer, 2005; Well-

Table 2. Changes of mRNA abundance of genes related to immune response in liver and udder tissue during infusion with BHBA (HyperB) or saline (NaCl) during a 48-h infusion

Parameter[1] and group[2]	Delta[3]	ANOVA (*P*-value, group)
Liver tissue		
TNFα		0.52
HyperB	0.4 ± 0.1	
NaCl	0.1 ± 0.4	
Hp		0.79
HyperB	3.6 ± 1.3*	
NaCl	4.0 ± 1.2*	
SAA		0.45
HyperB	2.5 ± 0.6*	
NaCl	1.7 ± 0.7*	
Udder tissue		
IL-1β		0.32
HyperB	−0.5 ± 0.4	
NaCl	0.4 ± 0.6	
IL-6		0.12
HyperB	0.6 ± 0.6	
NaCl	−0.4 ± 0.3	
IL-8		0.47
HyperB	0.6 ± 0.8	
NaCl	0.5 ± 0.8	
IL-10		0.17
HyperB	−0.3 ± 0.5	
NaCl	0.5 ± 0.3	
iNOS		0.45
HyperB	0.1 ± 0.3	
NaCl	2.0 ± 1.9	
TNFα		0.38
HyperB	0.7 ± 0.5	
NaCl	0.2 ± 0.3	
Lactoferrin		0.21
HyperB	0.6 ± 0.3	
NaCl	−0.1 ± 0.4	
NF-kB		0.64
HyperB	−0.4 ± 0.2	
NaCl	−0.1 ± 0.4	
Hp		0.07
HyperB	0.6 ± 0.4	
NaCl	−0.3 ± 0.2	
SAA		<0.01
HyperB	1.4 ± 0.5*	
NaCl	−0.7 ± 0.3	

[1]TNFα = tumor necrosis factor-α; Hp = haptoglobin; SAA = serum amyloid A; iNOS = inducible nitric oxide synthase; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells.

[2]HyperB = Hyper BHBA group; NaCl = group of cows receiving physiological saline solution.

[3]Delta = change from before the start of the infusion to 48 h after the start of infusions); values represent means ± SEM.

*Delta differs from 0 ($P < 0.05$).

nitz et al., 2011; Vernay et al., 2012). This result demonstrates that increased BHBA concentration in blood by infusion affects the abundance of measured genes related to immune response in the udder but not in the liver. Increased IL-6, IL-10, and Hp mRNA abundance in LPS quarters and control quarters in both treatment groups illustrated that intramammary LPS stimulation also affects the systemic immune response (Schmitz et al., 2004). Modified plasma BHBA concentration induced a more pronounced increase of IL-8 and IL-10 mRNA abundance compared with the control group in LPS-challenged quarters. Information about effects of hyperketonemia on mRNA abundance related to immune response is scarce. However, evidence shows that ketone bodies, specifically acetoacetate, increase IL-8 and IL-6 concentrations in human U937 and THP-1 monocyte cell lines and human umbilical vein endothelial cells, whereas the presence of BHBA does not affect these cytokine concentrations (Jain et al., 2007; Rains and Jain, 2011). Interleukin-8 is a chemokine produced by lymphocytes (Gregory et al., 1988), neutrophils (Strieter et al., 1990), and epithelial cells (Skansén-Saphir et al., 1993). It is involved in recruiting and activating (Harada et al., 1994) neutrophils during mastitis (Barber and Yang, 1998), and mammary epithelial cells secrete IL-8 in response to LPS (Wellnitz and Kerr, 2004). It can be hypothesized that increased IL-8 mRNA abundance after LPS challenge is related to its role in the recruitment of immune cells from blood into milk. As BHBA infusion decreased neutrophil recruitment, the immune system was most likely forced to compensate the deficiency of BHBA effects on immune cell recruitment through the upregulation of IL-8 mRNA abundance. It seems that upregulation of IL-8 and IL-10 in HyperB compared with the control group reflects the negative effects of BHBA on the immune response.

CONCLUSIONS

The results of the present study demonstrate that a long-term (48 h) elevation of plasma BHBA affects acute phase proteins in the mammary gland but not in the liver. The immune response to LPS challenge is clearly affected by elevated BHBA plasma concentration, which indicates that the frequently observed immunosuppression during spontaneous ketonemia in early lactation, and hence increased susceptibility of mastitis during this period, is most likely directly caused, at least in part, by the elevated concentration of BHBA.

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Table 3. Change of mRNA abundance of genes related to immune responses in mammary gland of cows infused with BHBA (HyperB) or saline (NaCl) during the LPS challenge in LPS and control quarters

Parameter[1] and group[2]	LPS Delta[3]	ANOVA (*P*-value, group)	NaCl Delta[3]	ANOVA (*P*-value, group)
IL-1β		0.28		0.59
HyperB	5.4 ± 0.9*		1.5 ± 1.1	
NaCl	2.9 ± 1.7*		0.7 ± 0.8	
IL-6		0.27		0.75
HyperB	5.8 ± 1.2*		2.2 ± 0.5*	
NaCl	4.3 ± 0.6*		2.5 ± 0.6*	
IL-8		<0.01		0.73
HyperB	10. 8 ± 0.6*		1.5 ± 0.7	
NaCl	6.5 ± 0.7*		2.1 ± 1.2	
IL-10		0.05		0.26
HyperB	4.6 ± 1.1*		2.2 ± 0.6*	
NaCl	2.3 ± 0.4*		1.2 ± 0.6*	
iNOS		0.47		0.78
HyperB	4.6 ± 0.5*		−0.4 ± 0.4	
NaCl	4.3 ± 2.6*		−1.7 ± 6.1	
TNFα		0.9		0.97
HyperB	2.7 ± 1.2*		0.5 ± 0.9	
NaCl	2.5 ± 0.7*		0.4 ± 0.6	
Lactoferrin		0.86		0.81
HyperB	1.4 ± 1.1		0.8 ± 0.8	
NaCl	1.1 ± 0.7		0.6 ± 0.5	
NF-κB		0.45		0.68
HyperB	1.7 ± 0.4*		0.1 ± 0.5	
NaCl	2.3 ± 0.6*		−0.2 ± 0.6	
Hp		0.97		0.15
HyperB	3.7 ± 0.6*		1.4 ± 0.6*	
NaCl	3.8 ± 1.1*		2.5 ± 0.4*	
SAA		0.78		0.34
HyperB	5.1 ± 1.3*		1.8 ± 1.6	
NaCl	5.6 ± 1.1*		3.4 ± 0.7*	

[1]iNOS = inducible nitric oxide synthase; TNFα = tumor necrosis factor-α; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; Hp = haptoglobin; SAA = serum amyloid A.

[2]HyperB = Hyper BHBA group; NaCl = group of cows receiving physiological saline solution.

[3]Delta = change from before the start of the infusion to 48 h after the start of infusions; values represent means ± SEM.

*Delta differs from 0 ($P < 0.05$).

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