Cytokine and acute phase protein gene expression in repeated liver biopsies of dairy cows with a lipopolysaccharide-induced mastitis

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ABSTRACT

A minimally invasive liver biopsy technique was tested for its applicability to study the hepatic acute phase response (APR) in dairy cows with *Escherichia coli* lipopolysaccharide (LPS)-induced mastitis. The hepatic mRNA expression profiles of the inflammatory cytokines, tumor necrosis factor α (TNF- α), IL-1 β , IL-6, and IL-10, and the acute phase proteins serum amyloid A isoform 3 (SAA3), haptoglobin (Hp), and α_1 -acid glycoprotein (AGP) were determined by real-time reverse transcription-PCR. Fourteen primiparous cows in mid lactation were challenged with 200 μ g of LPS ($n = 8$) or NaCl solution ($n = 6$) in 1 front quarter. Six repeated liver biopsies were collected at -22, 3, 6, 9, 12, and 48 h relative to LPS challenge in 4 LPS-infused cows and 3 NaCl-infused cows. The remaining cows had 3 liver biopsies taken at -22, 9, and 48 h. Production data and clinical signs were recorded and white blood cell counts and somatic cell counts (SCC) were analyzed to investigate the effect of repeated liver biopsies and verify the LPS model. Plasma concentrations of TNF- α , SAA3, Hp, and AGP were determined for comparison with the liver expression data. Repeated liver biopsies had no effects on the production data, clinical signs, or APR of dairy cows. Compared with the NaCl-infused cows the LPS-infused cows responded to the LPS treatment by increased body temperature (38.6 ± 0.1 vs. $39.4 \pm 0.1^\circ\text{C}$), short-term leukopenia followed by leukocytosis (6.44 ± 0.4 vs. $5.69 \pm 0.3 \times 10^6$ cells/mL), an increased SCC ($\log_{10} 2.1 \pm 0.1$ vs. $\log_{10} 2.8 \pm 0.1 \times 10^3$ cells/mL), heart rate (76 ± 1 vs. 93 ± 1 beats/min), and respiratory rate (32 ± 2 vs. 36 ± 1 breaths/min) in the acute phase of the disease. The LPS treatment upregulated the hepatic expression of TNF- α (103 ± 24 vs. 255 ± 18 units), IL-1 β (37 ± 23 vs. 296 ± 18 units), IL-6 (8 ± 17 vs. 122 ± 12 units), and IL-10 (130 ± 66 vs. 541 ± 50 units), and SAA3 (64 ± 36 vs. 128 ± 28 units) and Hp (9 ± 82 vs. 762 ± 65 units) reaching maximum levels at 3 to 6 h and 9 to 12 h postinfusion, respectively.

Plasma concentrations of TNF- α (nondetectable vs. 1.9 ± 0.3 ng/mL), SAA (19.8 ± 19.4 vs. 149.7 ± 15.5 μ g/mL) and Hp (71.4 ± 143.7 vs. $1,013.8 \pm 111.5$ μ g/mL) were elevated in the LPS-infused cows at 4 to 12 h, 8 to 120 h, and 24 to 120 h postinfusion, respectively. The hepatic expression of AGP and the AGP plasma concentration remained unaltered in LPS-induced cows. In conclusion, a minimally invasive liver biopsy technique can be used for studying the hepatic APR in diseased cattle. Lipopolysaccharide-induced mastitis resulted in a time-dependent production of inflammatory cytokines and SAA and Hp in the liver of dairy cows.

Key words: mastitis, acute phase response, liver biopsy, gene expression

INTRODUCTION

Lipopolysaccharide, a part of the gram-negative bacteria cell wall, is known as a potent inducer of inflammation and the acute phase response (APR; Berczi, 1998). Mastitis caused by *Escherichia coli* is a common disease in lactating dairy cows, and intramammary (IM) infusion with *E. coli* LPS is often used to simulate *E. coli* mastitis and can be used as a model for studying the bovine APR (Hoebe et al., 2000; Hiss et al., 2004; Lehtolainen et al., 2004). In vertebrates (mammals and birds), the APR is characterized by fever, leukocyte mobilization, an increased production of various inflammatory cytokines and acute phase proteins (APP), and profound hormonal and metabolic changes in which the liver plays a central role (Gruys et al., 2005).

From LPS studies conducted in inbred rodents (Sass et al., 2002; Ji et al., 2004), it is known that the liver is a major contributor of circulating inflammatory cytokines and that these have a local regulatory role in the hepatic APP synthesis. The proinflammatory cytokines tumor necrosis factor- α (TNF- α), IL-1 β , and IL-6 activate hepatocytic receptors and initiate the synthesis of APP such as serum amyloid A (SAA), haptoglobin (Hp), and α_1 -acid glycoprotein (AGP; Murata et al., 2004). Although the APR is highly conserved during evolution, some variations exist for the APP in terms of animal species, the inducing pathogen, pathogen dose, and site of infection (Murata et al., 2004). In cattle,

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limited information is available on the hepatic production of cytokines and APP and how these are regulated in LPS-induced mastitis. In vitro, LPS-treated bovine Kupffer cells (liver macrophages) are the major cytokine-producing cells (Yoshioka et al., 1998), and primary bovine hepatocytes secrete SAA and Hp in a time- and dose-dependent manner when stimulated with recombinant human proinflammatory cytokines (Yoshioka et al., 2002). In dairy cows, LPS administered IM increased the milk and plasma concentrations of TNF- α , SAA, and Hp, indicating both a local tissue production in the udder and a release of inflammatory cytokines and APP into the circulation (Hoebe et al., 2000; Hiss et al., 2004; Lehtolainen et al., 2004). Further, experimentally induced *Staphylococcus aureus* mastitis resulted in increased expression of Hp in liver tissue collected from cows killed 48 h postinfection (Eckersall et al., 2006). Sheep or near-term lambs given LPS intravenously had an acute upregulation of proinflammatory cytokines and SAA isoform 3 (**SAA3**) in liver tissue collected by biopsy (Kabaroff et al., 2006) or after killing (Wilson et al., 2005). Yet, in none of these studies were the kinetics of cytokine and APP in the liver of individual animals followed over time.

To study the kinetics of liver APR during inflammation in the same cow it is necessary to sample liver tissue using a minimally invasive biopsy technique. We have developed such a technique (Andersen et al., 2002), but even a minimally invasive biopsy technique involves local tissue damage that may induce an APR. Hence, to interpret results on the liver APR based on liver biopsies, the effect of repeated liver biopsies on APR must be investigated.

We hypothesized that an IM LPS challenge in cattle induces systemic cytokine and APP production in the liver and that the liver biopsy procedure used has a minimal effect on the APR and can be used for studying liver APR kinetics in vivo during a challenge or disease. To test this hypothesis, a study was set up to demonstrate the in vivo kinetics of cytokine and APP gene expression levels in liver biopsies from dairy cows with an IM inflammation experimentally induced with LPS. Further, the aim was to describe the relationship between TNF- α , SAA, Hp, and AGP mRNA levels in the liver and compare them with their concentrations in plasma.

MATERIALS AND METHODS

Animals and Treatment

All procedures involving animals were approved by the Danish Animal Experiments Inspectorate and complied with the Danish Ministry of Justice Laws

concerning animal experimentation and care of experimental animals.

Fourteen healthy, high-yielding (38 L/d, 4.2% fat) Holstein-Friesian dairy cows in first lactation were chosen. The udder health of the cows was evaluated based on SCC and bacteriological examinations. All cows had SCC <120,000/mL on both front quarters, and were free from mastitis pathogens. Nine to 12 wk after parturition, 8 cows were infused with LPS IM (LPS treatment) and 6 cows serving as negative controls were infused IM with a sterile 0.9% NaCl solution (NaCl treatment). For practical reasons the LPS challenge and the negative control study were conducted on 2 different dates.

Sterile polyvinyl catheters (Micro-Renathane, Braintree Scientific Inc., Braintree, MA) were inserted into the jugular vein the day before the LPS infusion. The catheters were flushed with a sterile 0.9% NaCl solution containing 50 IU/mL of Na-heparin (Loevens Kemiske Fabrik, Ballerup, Denmark) after each blood sampling.

Based on IM LPS studies performed by others (Blum et al., 2000; Hoebe et al., 2000) and a pilot study (data not shown), an intermediate LPS dose sufficient to induce a systemic TNF- α response in the dairy cows was chosen for the experiment. Each cow was given 200 μ g of *E. coli* LPS (0111:B4, Sigma-Aldrich, Brøndby, Denmark) dissolved in 10 mL of 0.9% pyrogen-free NaCl solution or 10 mL of 0.9% NaCl solution in the right front quarter. The treatments were given after the morning milking at time 0. Each teat was disinfected twice with cotton wool prewetted with 70% ethanol. The LPS or NaCl solution was infused into the gland with a sterile teat cannula and the quarter was thoroughly massaged.

One control cow exposed to 6 biopsies was excluded from the statistical analysis as it had abnormally high prechallenge plasma concentrations of SAA and Hp, indicating a concomitant disease (data not shown).

Feeding and Milking

The cows were housed in a traditional straw-bedded tie-stall barn, where they were fed individually and had free access to water. A TMR was fed ad libitum twice daily in equal portions at 0800 and 1400 h. The TMR consisted of grass silage (24%), barley silage (19%), corn silage (32%), rapeseed cake (11%), spring barley (6%), sugar beet molasses (3%), and barley straw (5%). The DM content of the diet was 50.3% (LPS challenge) and 45.8% (negative control). Further, each cow was fed 250 g of minerals and vitamins (Type 1, Vitfoss, Gråsten, Denmark) daily. The TMR diet and mineral supplementation were formulated to fulfill the nutrient requirements for dairy cows according to Danish

standards. The cows were milked at 0600 h and again at 1615 h. The daily feed intake (voluntary DMI) and milk yield were recorded.

Clinical Examinations and Sampling of Blood, Milk, and Liver

Systemic signs such as body temperature and white blood cell (WBC) count were recorded throughout the experiment. Clinical observations and body temperature were recorded at -42, -1, 2, 4, 6, 8, 10, 12, and 24 h relative to the time of LPS challenge. In addition, body temperature was recorded at 48, 57, and 72 h in the NaCl-infused cows. Two sets of blood samples were drawn from the catheters at -24, -1, 2, 4, 6, 8, 10, 12, 24, 33, 48, 57, 72, 96, and 120 h relative to the LPS infusion. One set of blood samples was collected in K₃EDTA tubes and analyzed for WBC count on a hemacytometer (Cell-Dyn 3500, Abbott Laboratories A/S, Copenhagen, Denmark). The other set was collected in Na-heparin tubes and placed on ice immediately after sampling.

Milk stripping samples were collected at -27, -3, 2, 4, 7, 12, 18, 21, 31, 45, 55, 69, 79, 93, 103, 117, and 127 h relative to the LPS and NaCl infusions. Immediately after sampling, 10 to 15 mL of milk was transferred to diagnostic SCC tubes preserved with dried 2-bromo-2-nitropropan-1,3-diol (bronopol; Eurofins Steins Laboratory, Holstebro, Denmark) resulting in a concentration of 0.01 to 0.02% bronopol in milk. The SCC analysis was performed as a fluoro-opto-electronic measurement (Fossomatic, Foss, Hillerød, Denmark; EN ISO 13366-3) in a diagnostic dairy laboratory. Pus and fibrin-like clumps in the milk at 21 h made it impossible to accurately measure SCC in the LPS-infused cows. Hence, the samples collected at 21 h for both the NaCl- and LPS-infused cows were excluded when performing the statistical analysis.

The liver biopsies were sampled as described by Andersen et al. (2002). Briefly, a skin area of 10 × 10 cm was shaved and disinfected with 70% ethanol. Then, local anesthesia was induced by injecting 10 mL of 2% lidocaine (Skanderborg Apotek, Skanderborg, Denmark) into the skin and underlying fat, muscular tissue, and fascia of the abdominal wall. After 10 min, a 0.5-cm-long incision was made in the skin. The liver biopsy was collected through the incision using a biopsy pistol (Manan Automatic Biopsy System, Marmon/MDTech, Gainesville, FL) developed for muscle tissue biopsies in humans. The needle of the biopsy pistol was 14 gauge with a 17-mm notch and collected tissue biopsies of about 20 mg. Because of ethical concerns, half of the cows were exposed to 6 biopsy samplings and the other half to only 3 samplings. Hence, 4 LPS-infused cows

and 3 control cows were sampled at -22, 3, 6, 9, 12, and 48 h relative to LPS or NaCl infusion, and 4 LPS-infused cows and 3 control cows were sampled at -22, 9, and 48 h relative to LPS or NaCl infusion. A new skin incision was made 1 to 1.5 cm above the former for each repeated biopsy. After the biopsies had been collected, each skin incision was closed with a single-use metal skin staple (35W Auto Suture, Appose ulc, Tyco Healthcare UK Ltd., Gosport, UK) and sprayed with a disinfecting wound spray (Tar Plaster, Jørgen Kruuse A/S, Marstal, Denmark).

Sample Preparations

Plasma was harvested (2,000 × g, 4°C) from the second set of blood samples within 30 min of sampling and stored at -80°C until analyzed. Liver biopsies for RNA extraction were immediately frozen in liquid nitrogen and transported to the laboratory where the biopsies were stored at -80°C until RNA extraction. Total RNA was extracted from liver biopsies of the LPS-infused cows using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA from biopsies obtained from the NaCl-infused cows was harvested on a later occasion using a phenol-chloroform extraction and phase Lock Gels (Heavy 2.0 Eppendorf, Hamburg, Germany) as described by the manufacturer. Concentrations and quality of total RNA dissolved in Tris-EDTA buffer (10 mM Tris-HCl, pH 8) were measured on a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Hillerød, Denmark). If the optical density (OD) absorption ratio OD_{260nm}/OD_{280nm} was >1.9, samples were adjusted to a final concentration of 0.2 µg/µL, which was subjected to reverse transcription; otherwise, total RNA was extracted again from a second set of tissue samples.

Reverse Transcription of Total RNA

Single-stranded cDNA was synthesized using the High-Capacity cDNA Archive Kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Briefly, reverse transcription (RT) was set up manually in a 100-µL final volume containing 10 µg of total RNA, RT buffer, dNTP mixture, random primers, MultiScribe reverse transcriptase (50 U/µL), and RNase-free H₂O. The mixture was subjected to 25°C for 10 min and 37°C for 120 min. The cDNA was analyzed immediately or stored at -80°C until use.

Primers and TaqMan Probes

Specific primers and probes were designed for each target gene using the Primer Express software (PE Ap-

plied Biosystems). The sense and antisense primers were placed in 2 consecutive exons of the gene. The probe spanned the junction of 2 exons, covered by the forward and reverse primers, to ensure discrimination between cDNA and genomic DNA. The bovine sequences for Hp and AGP were not available in GenBank at the time of the experiment. Thus, the bovine sequence was found by a BLASTn search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the human sequence. Pieces of bovine sequences found by the BLASTn search were aligned to create one consensus. This consensus was then aligned to the human sequence to deduce intron-exon boundaries, based on the assumption that the positions of introns are highly conserved among species. Only junctions with great homology (>90%) between the human and the bovine sequences were chosen for probe design. Since then, both the Hp and AGP sequences have been reported (from the Ensembl homepage <http://www.ensembl.org/index.html>; numbers ENSBTAT00000008339 and ENSBTAT00000022991, respectively), and these new sequences corresponded to the consensus sequence obtained from the BLASTn search of the human sequences.

Each probe was labeled at the 5'-end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3'-end with a nonfluorescent quencher and a minor groove binder, which increased the melting temperature without increasing the probe length. All primers and probes were synthesized by PE Applied Biosystems. Detailed information regarding primer and probe sequences, melting temperature, and PCR product length used for real-time quantitative PCR is in Table 1.

Real-Time TaqMan PCR for Quantitation of cDNA

Reverse transcription and real-time PCR was performed in 2 steps. Equal amounts of the synthesized bovine cDNA (1 µg) were assayed for each of the cytokine and APP profiles in separate wells. All samples were evaluated in triplicate. The real-time PCR reaction was performed in 96-well plates with a final volume of 50 µL. The PCR mixture contained a diluted cDNA sample, 900 nM of each primer, 250 nM of the TaqMan probe, and commercially available Mastermix (TaqMan Universal PCR Mastermix, PE Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTP with dUTP, the passive reference dye ROX, and optimized buffer components. Samples and PCR mixture were loaded onto a 96-well plate and amplified in an automated ABI Prism 7900 HT Sequence Detection System (PE Applied Biosystems) equipped with Sequence Detection System software version 2.1 (PE Applied Biosystems). For all assays, amplification conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Specificity of the assays was evaluated by using total RNA (without the RT step) as template for the real-time PCR, thereby verifying that there was no amplification of cDNA. Furthermore, amplified PCR products were subjected to gel electrophoresis on a 3% MetaPhor Agarose (Cambrex, Copenhagen, Denmark) gel to validate the size of the PCR products. Each plate included a nontemplate control to verify contamination of assay reagents. All samples were tested in triplicate and the mean was obtained for further calculations. Relative quantification was done using the relative standard curve method, and results are reported as relative mRNA abundance.

Relative Standard Curve Method

The relative standard curve method was based on a stock cDNA sample containing the appropriate target of interest. From this sample, a 2-fold serial dilution was made to make a standard curve. For all experimental samples, target quantity was determined from the standard curve and the target quantity was expressed in arbitrary units. It is common practice to normalize the target gene expression results to a reference gene that is stable and unaffected by the experimental treatment (De Ketelaere et al., 2006). In our study, the expression of GAPDH and 28S in the liver biopsies of LPS-infused cows were affected by the LPS treatment (data not shown). Thus, the target gene expression was normalized to the concentration of total RNA, as described by Bustin (2000) as being the most reliable normalization method.

TNF- α , SAA, and Hp ELISA

Plasma concentration of TNF- α was determined by ELISA as described in Røntved et al. (2005). This ELISA was modified to quantify bovine TNF- α in plasma by adjusting for the matrix effect of plasma. The standard consisted of bovine recombinant TNF- α (Ciba-Geigy, Basel, Switzerland; 62.5 ng/mL) diluted 2-fold in 50% Tris-buffered saline (0.05 M Tris, 0.15 M NaCl, pH 7.6) containing 0.05% Tween and 0.5% gelatin (Sigma-Aldrich) and 50% bovine heparin-stabilized plasma (pool) found below the detection limit of the TNF- α ELISA. Heparin-stabilized plasma harvested from whole bovine blood stimulated for 3.5 h with 5 µg/mL *E. coli* LPS (O111:B4) served as positive controls. The plasma pool used for the standard served as a negative control. The plasma samples and controls were diluted 1:2 in Tris-buffered saline-Tween-gelatin and tested in duplicate. Standard and controls were added to each plate and all samples were assessed on the same day. The intra- and interassay coefficients of variation (CV) were <6.3%

Table 1. Sequences of PCR primers and TaqMan probes, predicted primer melting temperature (T_m) and PCR product length for the specific bovine cytokines and acute phase proteins

Gene specificity ¹	Primer	Primer sequence (5' – 3')	T_m (°C)	PCR product length (bp)	Probe sequence (5' – 3') ²	Accession number ³
TNF- α	Forward	CCTCTTCTCAAGCCTCAAGTAACAA	59.1	69	CACGTTGTAGCCGACATC	Z141337
	Reverse	GCTGCCCCGGAGAGTT	55.6			
IL-1 β	Forward	GCTCTCCACCTCCTCTCACA	56.5	69	ACCACTTCTCGGTTTCATTT	NM_174093 ⁴
	Reverse	CTCTCCTTGCACAAAGCTCATG	58.7			
IL-6	Forward	AGGACGGATGCTTCCAATCTG	60.0	66	CAATCAGGCGATTTGC	NM_173923 ⁴
	Reverse	GAAGACCAGCAGTGGTTCTGAT	57.0			
IL-10	Forward	CAAGGAGCACGTGAACCTCACT	56.7	92	CTGCGGCGCTGTCAT	NM_174088 ⁴
	Reverse	TCTTGTTTTTCGCAGGGCAGAA	61.7			
SAA3	Forward	GGTGCCCTGGGCTGCTAA	56.8	62	TCTGGCGTTACTGATCACT	AF160867 ⁴
	Reverse	GGGTCTGTGATTCCCTGAATAGTCT	59.7			
Hp	Forward	AACATTGGACAGAAACTCCCTGAAT	60.1	93	CCCGCACACTGCCTC	
	Reverse	CAATGAGCCACCGATGATCCT	60.8			
AGP	Forward	AGTGGCGCCCATTACCAAT	58.9	66	CCACCATGGACTTGCT	
	Reverse	GCGGAGCCAATATAAAACCACTTG	61.7			
GAPDH	Forward	GCCGTGGGCAAGGTCAT	59.0	92	CTCAACGGAAGCTCA	AF077815
	Reverse	GTCAGATCCACAACAGACACGTT	57.7			
28S	Forward	GATTTTGGCGGCAAGTTTCATTG	63.4	81	TTTCTCTCCATCCACTTCCG	AF154866
	Reverse	CCGACTCTGGTTCCTTCTG	59.7			

¹TNF- α = tumor necrosis factor α ; SAA3 = serum amyloid A, isoform 3; Hp = haptoglobin; AGP = α_1 -acid glycoprotein.

²Exon-exon junctions are underlined.

³If no accession number, the bovine sequence was found by a BLASTn search of the human sequence. The bovine sequence pieces were aligned to create one consensus. This consensus sequence was aligned to the human sequence to identify intron-exon boundaries. Only junctions with great homology between the human and the bovine sequences were chosen for probe design.

⁴As the genomic sequence for bovine SAA3 and some of the cytokine genes were not available, intron-exon junctions were deduced from human sequences based on the assumption that the position of introns was highly conserved among species.

for the low (1.4 ng/mL) and high (5.6 ng/mL) positive controls, respectively. The detection limit of the ELISA was 0.5 ng/mL for bovine plasma.

Plasma concentrations of SAA were measured by a commercially available ELISA kit (Tridelta Development Ltd., Bray, Co. Wicklow, Ireland) based on methods described by McDonald et al. (2001) and performed according to the manufacturer's instructions. The plasma samples were initially diluted 1:500 and tested in duplicate. Test samples outside the range of the standard curve were diluted further in dilution buffer and reanalyzed. Maximum dilution of plasma samples was 1:4,000. The in-house detection limit of the assay was 4.7 µg/mL. The interassay CV was <16.8% and the intraassay CV was <10% for the low (56.4 µg/mL) and high (260.0 µg/mL) positive controls.

The plasma concentration of Hp was measured by using a commercially available solid-phase ELISA kit (Tridelta Development Ltd.) according to the manufacturer's instructions. The plasma samples were diluted 1:500 and tested in duplicate. Samples falling outside the range of the standard curve were further diluted in dilution buffer and reanalyzed. The maximum dilutions of plasma samples were 1:8,000. The in-house detection limit of the assay was 10.6 µg/mL. The interassay CV was <15% and the intraassay CV was <2% for the low (58.2 µg/mL) and high (195.3 µg/mL) positive controls.

AGP Single Radial Immunodiffusion

The concentration of AGP in plasma was semiquantified by a single radial immunodiffusion, using a commercially available kit (Tridelta Development Ltd.). According to the manufacturer's instructions, a high (A: 1,000 µg/mL) and a low (B: 250 µg/mL) standard were included on each of the gels. The plates were incubated for 44 h, whereupon the diameters of the precipitin rings were measured using the precipitin ring measurement scale. The diameters from the 2 standards were plotted to make a reference curve. The interassay CV of the standards on 14 gels was <1% for A (9.0 mm) and B (5.6 mm). From the reference curve, AGP concentrations of each undiluted test sample were calculated. The sizes of the plasma sample precipitation rings were between the sizes of the 2 standards.

Statistical Analysis

The effect of IM LPS challenge or NaCl infusion on repeated measurements of cytokine and APP gene expression in liver biopsies and plasma concentrations of TNF-α, SAA, Hp, and AGP, and DMI and milk yield,

were statistically analyzed using the MIXED procedure in SAS (SAS Institute, 1999) and the following model:

$$Y_{ijkt} = \mu + \alpha_i + \delta_j + (\alpha\delta)_{ij} + A_k + \gamma_t + (\alpha\gamma)_{it} + \varepsilon_{ijt},$$

where Y_{ij} = dependent variable; μ = least squares mean; α_i = fixed effect of treatment i (i = LPS solution, NaCl solution); δ_j = fixed effect of number of biopsies j per cow (j = 3, 6); $(\alpha\delta)_{ij}$ = fixed effect of the interaction between treatment and number of biopsies; A_k = random effect of cow k (k = 1, 2, . . . 13) and $\{A_j\} \sim N(0, \sigma_A^2)$; γ_t = fixed effect of time t from infusion of LPS or NaCl solution: for clinical variables: (t = -42, -1, 2, 4, 6, 8, 10, 12, and 24 h from infusion; body temperature also at 48, 57, and 72 h in the NaCl-treated cows); for blood variables: (t = -24, -22, 2, 3, 4, 6, 8, 9, 10, 12, 24, 33, 48, 57, 72, 96, 120 h from infusion); for liver tissue variables: (t = -22, 3, 6, 9, 12 and 48 h from infusion); for milk variables: (t = -27, -3, 2, 4, 7, 12, 18, 21, 31, 45, 55, 69, 79, 93, 103, 117, and 127 h from infusion); and ε_{ijt} = random error; $\{\varepsilon_{ijt}\} \sim N(0, \sigma^2)$. A first-order autoregressive covariance structure was defined to take into account significant autocorrelation between measurements within cow. All values are reported as least squares means $\pm$ standard errors of the mean.

RESULTS

An overview of the P -values from the statistical analysis of treatments (LPS vs. NaCl), and biopsies (3 vs. 6 times), time relative to infusion, and the interactions between treatment versus biopsies and interactions between treatments versus time relative to infusion are in Table 2.

Effect of Liver Biopsy Sampling

Dry matter intake, daily milk yield, clinical signs, WBC, cytokine, and APP responses did not change in the NaCl-infused cows due to repeated liver biopsies (Figures 1, 2, 3, 4, 5, and 6). No differences were found between cows biopsied 3 versus 6 times with regard to DMI, clinical signs (heart rate, respiratory rate), WBC, SCC, or cytokine and APP responses. Sampling of 6 biopsies resulted in a 13.4% lower (33.1 vs. 38.2 L) daily milk yield compared with sampling of 3 biopsies; however, this effect was due to the random placement of 2 relatively low-yielding cows on the 6-biopsy treatment compared with the 3-biopsy treatment. Also, the biopsy treatment did not alter the daily milk yield of the NaCl-infused cows (Figure 1C). Finally, cows exposed to 6 biopsies compared with 3 biopsies tended

Table 2. Probability values of main effects and interactions of cows infused with LPS¹

Trait ²	Treatment	Biopsy	tINF	Treatment × biopsy	Treatment × tINF
Production data					
DMI	0.18	0.90	<0.0001	0.13	<0.0001
Milk yield	0.03	0.02 ³	<0.0001	0.002 ⁴	<0.0001
Clinical signs					
Body temperature	<0.0001	0.07	<0.0001	0.30	<0.0001
Heart beat rate	<0.0001	0.30	<0.0001	0.93	<0.0001
Respiratory rate	0.11	0.94	<0.0001	0.02	0.004
Milk trait					
SCC	0.008	0.42	<0.0001	0.04	<0.0001
Blood trait					
White blood cells	0.16	0.90	<0.0001	0.30	<0.0001
Neutrophils	0.83	0.31	<0.0001	0.32	<0.0001
Monocytes	0.25	0.07	<0.0001	0.59	<0.0001
Lymphocytes	0.05	0.54	<0.0001	0.05 ⁴	<0.0001
TNF- α , ng/mL	ND ⁵	0.91	<0.0001	ND	ND
SAA, μ g/mL	<0.0001	0.56	<0.0001	0.93	0.003
Hp, μ g/mL	0.0005	0.79	<0.0001	0.91	<0.0001
Liver mRNA expression					
TNF- α	<0.0007	0.29	0.0009	0.97	0.04
IL-1	<0.0001	0.92	<0.0001	0.87	<0.0001
IL-6	0.0008	0.24	0.006	0.84	0.01
IL-10	0.002	0.99	0.0002	0.64	0.0002
SAA3	0.27	0.49	0.0006	0.81	0.0006
Hp	0.05	0.94	<0.0001	0.92	<0.0001

¹Treatment: LPS-infused cows vs. NaCl-infused cows; biopsy: cows exposed to 3 biopsies vs. cows exposed to 6 biopsies and time relative to infusion (tINF): Interactions are shown for treatment vs. biopsy and treatment vs. tINF.

²TNF- α = tumor necrosis factor α ; SAA3 = serum amyloid A, isoform 3; Hp = haptoglobin.

³This effect was due to the random placement of 2 relatively low-yielding cows on the 6 biopsy treatment compared with the 3 biopsy treatment.

⁴This effect was due to the random placement of LPS-infused cows with a normal but lower lymphocyte count before LPS-infusion on the 6 biopsy treatment compared with the 3 biopsy treatment.

⁵ND = not detectable; the TNF- α concentration in the plasma samples of the NaCl-infused cows was below the detection limit of the assay.

to have a 0.2°C higher body temperature and 5% lower monocyte count (data not shown).

Effect of LPS on Production, Clinical, and Hematological Data

Intramammary LPS infusion resulted in a decreased DMI and milk yield in cows in the first 48 h following the challenge, whereas the NaCl-infused cows maintained their DMI and milk yield throughout (Figure 1). Local clinical symptoms, such as quarter swelling and warmth, and altered milk appearance began 4 h after LPS infusion (data not shown) concomitantly with an increased SCC that reached maximum levels 12 h after the LPS infusion (Figure 2a). Further, a significant decrease in WBC was observed 4 to 10 h after LPS infusion that was followed by an increased WBC at 33 to 48 h (Figure 2b). The significant changes in WBC counts (Table 2) were because of a decreased number of neutrophils, monocytes, and lymphocytes in the early phase and an increased number of neutrophils and monocytes in the late phase (data not shown). Infusion of LPS resulted in the temperature reaching 41.5°C at

6 h and increased heart and respiratory rates at 6 to 12 h (Figure 3). The NaCl infusion did not cause any local or systemic clinical symptoms or changes in WBC (Figures 2 and 3). An increase of 0.5°C was observed in the NaCl-infused cows at 10 to 12 h, but this increase was considered to be within the normal diurnal variation. Furthermore, body temperature was recorded for the NaCl-infused cows for the following 48 h when the average body temperature remained below 39°C (Figure 3A). Significant increases in the average SCC were found at some time points in the NaCl-infused cows, but the level always remained less than in the LPS-infused cows.

Effect of LPS on Hepatic Gene Expression of Cytokines and APP

Gene expression analysis of liver biopsies of LPS-infused and NaCl-infused cows was performed on 2 separate occasions. However, selected cDNA samples from the LPS-infused cows were reanalyzed simultaneously with the cDNA samples from the NaCl-infused cows. This confirmed that there were no differences in

Figure 1. Daily DMI (A) and daily milk yield (B) of cows infused with LPS (closed triangle, $n = 8$) and NaCl (open triangle, $n = 5$) relative to the time of the LPS or NaCl infusion (tINF, h); (C) daily milk yield of individual NaCl-infused cows exposed to 3 (open symbols) or 6 (closed symbols) biopsies during the study. Results are expressed as least squares means $\pm$ SEM. Asterisks indicate the differences of the LPS-treated cows compared with their prechallenge values; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

expression levels between the 2 batches of analysis and, consequently, that it was possible to compare expression levels between the NaCl- and the LPS-infused cows.

Treatment with LPS upregulated gene expression in the liver of all measured cytokines (Figure 4), SAA3, and Hp (Figure 5), but not AGP (data not shown). Tumor necrosis factor- α and IL-1 β responded rapidly, being significantly increased at 3 to 9 h, with peak expression values 3 h after challenge. Furthermore, the

TNF- α mRNA level was significantly increased at 48 h compared with the prechallenge level. Expression levels of IL-6 and IL-10 mRNA were significantly elevated at 3 to 9 h after challenge, with peak expression values 6 h after challenge. After 48 h, IL-1 β , IL-6 and IL-10 mRNA levels were back to prechallenge (-22 h) levels. At peak level, TNF- α was upregulated 3-fold, IL-1 β 4-fold, IL-6 75-fold, and IL-10 13-fold compared with the expression levels before challenge.

Expression of SAA3 mRNA was significantly elevated 3 to 48 h after challenge compared with prechallenge, with peak expression at 9 h postchallenge. Haptoglobin mRNA was significantly increased 3 to 48 h after challenge, gradually increasing to a peak at 12 h. At their peaks, SAA3 was upregulated 145-fold and Hp 71-fold. The expression of AGP mRNA was not significantly altered during the LPS challenge (data not shown); hence, AGP mRNA was not measured in the NaCl-infused cows. The expression levels of cytokine and APP genes in liver did not change over time in the NaCl-infused cows.

Effect of LPS on TNF- α , SAA, Hp, and AGP Concentrations in Plasma

The LPS infusion caused a significant increase in plasma concentrations of TNF- α , SAA, and Hp compared with the prechallenge levels and compared with concentrations in the NaCl-infused cows (Figure 6). Before the LPS challenge, only 2 LPS-infused cows had measurable levels of plasma TNF- α . After the LPS challenge, the plasma concentration of TNF- α was significantly increased after 4 h, reaching maximum concentrations after 6 h, and decreasing to preinfusion levels at 24 h after which plasma TNF- α was undetectable. In the NaCl-infused cows, TNF- α plasma concentrations were below the detection limit. Concentrations of SAA started to increase 8 h after the LPS challenge until a plateau was reached at 24 to 57 h, which was followed by a decrease until the last sampling at 120 h. Haptoglobin in plasma was not detectable until 10 h after the LPS challenge. From 12 h onward, Hp concentration increased until a plateau was reached at 48 to 72 h after the LPS challenge, which was followed by a decrease until the last sampling at 120 h. At their peak, the mean TNF- α concentrations were 7 times greater, the mean SAA 16 times greater, and the mean Hp 134 times greater (from the detection limit of the Hp ELISA) compared with the samples taken before the LPS challenge. In the NaCl-infused control cows, the average mean levels of SAA and Hp concentrations slowly increased by the end of the study. But these increases were not significantly different from prechallenge levels and were due to an increased concentration

Figure 2. Somatic cell count (A) and white blood cell count (WBC) (B) of LPS (closed triangle, $n = 8$) and NaCl (open triangle, $n = 5$) infused cows relative to the time of the LPS or NaCl infusion (tINF). Results are expressed as least squares means $\pm$ SEM. Asterisks indicate the differences of the LPS-treated cows compared with their prechallenge values; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

in the same 2 cows. The mean plasma AGP concentration of the LPS-infused cows before the LPS challenge was $458 \pm 86 \mu\text{g/mL}$, ranging from 402 to $477 \pm 86 \mu\text{g/mL}$, after the LPS challenge and was not significantly different (data not shown). Hence, it was decided not to measure AGP in the NaCl-infused cows.

DISCUSSION

To our knowledge, this is the first study showing that a minimally invasive liver biopsy procedure performed repeatedly in dairy cows can be used to study APR kinetics in vivo during challenge or disease without affecting the results. Comparing the NaCl-infused control cows with their own prebiopsy levels and with LPS-infused cows showed that repeated liver biopsy sampling (within 72 h) had no effect on systemic APR, clinical signs, DMI, or daily milk yield. Moreover, no significant differences were found in cytokine and APP expression levels between cows sampled 3 versus 6 times. Thus, in agreement with our hypothesis, the minimally invasive technique used to collect liver biopsies in dairy cows

Figure 3. Body temperature (A), heart rate (B), and respiratory rate (C) of LPS (closed triangle, $n = 8$) and NaCl (open triangle, $n = 5$) infused cows relative to the time of the LPS or NaCl infusion (tINF). Asterisks indicate the differences of the LPS-treated or NaCl-treated cows compared with their prechallenge values; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

was an acceptable method for studying the liver APR kinetics in vivo.

In the present study, IM LPS infusion in dairy cows induced a significant APR in the liver. This was observed as significant changes in the kinetics of in vivo cytokine and APP mRNA expression in the liver. The LPS model was verified by the clinical and paraclinical findings, including increased body temperature (fever), short-term leukopenia followed by leukocytosis, increased SCC, and increased plasma concentrations of

SAA and Hp. These are all characteristic findings in an IM LPS model (Hiss et al., 2004; Lehtolainen et al., 2004). Moreover, the dose of 200 μ g of LPS induced a systemic plasma TNF- α response peaking at 6 h, which is in agreement with previous findings (Blum et al., 2000; Hoeven et al., 2000). Thus, we have shown that gene expression of the proinflammatory cytokines TNF- α , IL-1 β , IL-6, and IL-10 and APP, SAA3, Hp, and AGP could be measured quantitatively by a real-time RT-PCR assay of repeated liver biopsies, suggesting that the liver contributes to the overall pool of circulating cytokines and APP during mammary inflammation. As no changes were found in the NaCl-infused cows with regard to production data, clinical signs, and inflammatory measures, this study clearly demonstrates that IM LPS infusion induced a systemic cytokine and APP production in the liver. Moreover, as sampling was done repeatedly, the mRNA expression of the proinflammatory cytokines TNF- α and IL-1 β peaked simultaneously and were the fastest responding cytokines in the liver, followed by IL-6 and the antiinflammatory cytokine IL-10, which were expressed concomitantly following the same kinetic pattern. In LPS studies conducted on mice injected i.p. with LPS (Sass et al., 2002; Zhong et al., 2006), the expression of IL-6 and IL-10 peaked earlier and simultaneously with

the expression of TNF- α and IL-1 β in the liver. But differences in the LPS application route, LPS dose, animal species, and time of sampling possibly contributed to the different liver cytokine profiles in LPS-treated dairy cows.

In addition to the upregulated genes of inflammatory cytokines in the liver, there was a significant increase in plasma TNF- α concentration 4 to 6 h after LPS challenge simultaneously with the fever response and mobilization of blood leukocytes to the mammary gland, supporting the idea of proinflammatory cytokines acting as inducers of the APR. Several studies have investigated the kinetics of bovine plasma concentrations of SAA (Lehtolainen et al., 2004) and Hp (Jacobsen et al., 2004) in response to LPS. In our study, the time-related changes were investigated in the amount of liver mRNA coding for SAA3, Hp, and AGP as a response to LPS-induced mastitis. The SAA3 gene was upregulated in the liver at the same time as the Hp gene (Figure 5), which was reflected in the plasma concentrations where SAA was increased 8 h after challenge, whereas the Hp concentration was increased after 12 h. These plasma changes are in agreement with other studies reporting plasma concentration kinetics, where SAA responds faster than Hp (Jacobsen et al., 2004). Although not shown in LPS models, AGP is considered an APP in

Figure 4. The mRNA expression of A) tumor necrosis factor α (TNF- α), B) IL-1 β , C) IL-6, and D) IL-10 in liver tissue at times relative to LPS challenge (tINF) of LPS (white bar) and NaCl (gray bar). Results are expressed as least squares means $\pm$ SEM. Asterisks indicate the differences of the LPS-treated cows compared with their values at tINF = -22; * P < 0.05, ** P < 0.01, *** P < 0.001. Liver biopsies collected at tINF = -22, 9, 48, n = 8 (LPS) and n = 5 (NaCl), whereas liver biopsies collected at tINF = 3, 6, and 12, n = 4 (LPS) and n = 2 (NaCl).

Figure 5. The mRNA expression of A) serum amyloid A isoform 3 (SAA3), and B) haptoglobin (Hp) of cows injected with LPS (white bar) and NaCl (gray bar) relative to the time of the LPS or NaCl infusion (tINF). Results are expressed as least squares means $\pm$ SEM. Liver biopsies collected at tINF = -22, 9, 48, n = 8 (LPS) and n = 5 (NaCl), whereas liver biopsies collected at tINF = 3, 6, and 12, n = 4 (LPS) and n = 2 (NaCl). Asterisks indicate the differences of the LPS-treated cows compared with their values at tINF = -22; * P < 0.05, ** P < 0.01, *** P < 0.001.

cattle (Eckersall et al., 2001; Murata et al., 2004). In our study, AGP was present in the plasma of all cows before the LPS infusion in similar plasma concentrations as described for healthy cows in Eckersall et al. (2001). However, no changes were found in the LPS-infused cows either in the gene expression of AGP mRNA in the liver or the AGP plasma concentration compared with prechallenge levels.

Taking both the cytokine and the APP kinetic results into consideration, our findings support the general understanding that a severe local inflammation in a peripheral tissue results in the induction of proinflammatory cytokines in the liver (TNF- α , IL-1 β , IL-6), which is accompanied by the antiinflammatory cytokine IL-10 (Zhong et al., 2006) and the hepatic synthesis of APP (Murata et al., 2004). Still, how the proinflammatory cytokines induce the synthesis of APP in the bovine liver and to what extent this is modulated by antiinflammatory cytokines in dairy cows needs further investigation.

Comparing the average mRNA levels with the average protein levels resulted in a time-lag from peak mRNA to peak protein of 3 h for TNF- α , 39 h for SAA, and 36 h for Hp. Some kind of time-lag was expected

based on the time from upregulation of the gene until its synthesis by the liver.

As the SAA isoform specificity of the ELISA is unknown and SAA3 mRNA was the isoform measured in the liver, it is likely that the isoforms SAA1 and SAA2 also contributed to the prolonged and elevated SAA concentration measured in plasma (Wilson et al., 2005). Further, the induction of extrahepatic SAA (McDonald et al., 2001) and Hp (Hiss et al., 2004) in other major compartments during the APR may have contributed to APP the circulation.

The reported half-lives in plasma are between 30 min and 2 h for SAA in mice (Tape and Kisilevsky, 1990) and 4.5 d for the Hp protein in humans (Dobryszczyka, 1997). Nevertheless, the turnover of the proteins may change during the APR, can be accelerated in certain disease states, and may differ between species. Thus, long or increased half-lives of the APP may be another factor contributing to the time-lag between mRNA and protein peaks. In contrast to the APP, TNF- α was not measurable in the circulation after 24 h, although hepatic gene expression of TNF- α was still increased at 48 h. This suggests the presence of TNF- α binding molecules in the circulation, such as the soluble TNF- α receptor I and II (Bemelmans et al., 1996) that bind or inactivate TNF- α , making it undetectable in plasma by ELISA.

Although the main immunological functions of APP are still not understood, the APP are believed to contribute to host defense by decreasing tissue damage, acting on leukocyte functions, inhibiting bacterial activity through binding of endotoxin, and displaying antiinflammatory properties (Moore et al., 1997; Murata et al., 2004). Thus, the APP are important in the nonspecific immune system. α_1 -Acid glycoprotein is present at high concentrations in the blood of healthy individuals compared with SAA and Hp. Hence, AGP might exert its properties in the early phase of the APR or before the APR. In contrast, SAA is only found in very small concentrations and Hp is not detectable in the blood of healthy individuals, but the blood concentration of both proteins increases dramatically after LPS infusion, with SAA appearing before Hp. This suggests that SAA may play its role in the acute and middle phase of the APR, whereas Hp plays its role in the late phase of APR, when it reaches much greater blood concentrations than either AGP or SAA.

In conclusion, repeated liver biopsies had no effect on DMI, daily milk yield, clinical signs, or liver expression of the tested cytokine and APP genes. Consequently, this minimally invasive fine needle technique can be used for studying the function of the liver in the APR in diseased cattle. Using an IM LPS challenge, the liver expressed both inflammatory cytokines and APP in a

Figure 6. The blood concentration of A) tumor necrosis factor α (TNF- α), B) serum amyloid A (SAA), and C) haptoglobin (Hp) of cows infused with LPS (white bar) and NaCl (gray bar) relative to the time of the LPS and NaCl infusion (tINF). Results are expressed as least squares means $\pm$ SEM. Liver biopsies collected at tINF = -22, 9, 48, n = 8 (LPS) and n = 5 (NaCl), whereas liver biopsies collected at tINF = 3, 6, and 12, n = 4 (LPS) and n = 2 (NaCl). Asterisks indicate the differences of the LPS-treated cows compared with their prechallenge values; * P < 0.05, ** P < 0.01, *** P < 0.001.

time-dependent manner in response to the LPS before and during the presence of TNF- α , SAA, and Hp in the circulation.

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