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Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis

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Abstract

During mammary gland infection, non-specific responses are the predominant ones. The goal of this study was to investigate the mRNA expression of various soluble immune components and of the major milk proteins during the acute phase of mammary inflammation. Five healthy lactating cows were intramammary infused in one quarter with 100 μg *Escherichia coli*-endotoxin (lipopolysaccharide, LPS) and the contralateral quarter with saline (9 g/l) serving as control. Mammary biopsy samples of both quarters were taken immediately before and at 3, 6, 9 and 12 h after infusion and mRNA expression of various factors was quantified via real-time RT–PCR. Blood samples for determination of leukocyte number were taken simultaneously with the biopsy samples and rectal temperature was measured at 1-h intervals. Rectal temperature increased until 5 h ($P < 0.05$) after LPS administration and remained elevated until 9 h after LPS inoculation. Blood leukocyte number decreased ($P < 0.05$) from 0 to 3 h from $7.7 \pm 1.1 \times 10^9$ l^{-1} to $5.7 \pm 1.0 \times 10^9$ l^{-1} and thereafter recovered to pre-treatment levels until 12 h after LPS challenge. In LPS-treated quarters, tumor necrosis factor-α and cyclooxygenase-2-mRNA expression increased ($P < 0.05$) to highest values at 3 h after LPS challenge. Lactoferrin, lysozyme, inducible nitric oxide synthase increased ($P < 0.05$) and peaked at 6 h after challenge, and platelet-activating factor acetylhydrolase-mRNA expression tended to increase ($P = 0.07$). mRNA expression of insulin-like growth factor-I and of αS1-casein (CN), αS2-CN, β-CN and β-lactoglobulin did not change significantly, whereas mRNA expression of 5-lipoxygenase and α-lactalbumin decreased ($P < 0.05$) in both quarters and that of κ-CN only in the LPS quarter. mRNA expression of some investigated factors (tumor necrosis factor-α, lysozyme, 5-lipoxygenase, α-lactalbumin) changed in control quarters, however in all respective factors less than in the LPS quarters ($P < 0.05$). In conclusion, mRNA expression of most inflammatory factors increased within hours, whereas that of most milk proteins remained unchanged.

Keywords: LPS challenge; Mastitis in cow; Inflammatory factors

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1. Introduction

The mammary gland is protected by a variety of defence mechanisms, most of them acting as non-specific immune response by leukocytes and by soluble immune components such as inflammatory mediators and antimicrobial factors, which are readily available at the site of infection. Exposure of a tissue to *Escherichia coli* endotoxin is known to result in a rapid inflammation as a model to study the inflammatory response of the cow's udder. To elicit the neutrophil recruitment into the udder, characterizing mammary immune response, various inflammatory mediators are secreted [1]. Besides chemotactic activity on leukocytes inflammatory mediators like arachidonic acid metabolites and cytokines are also able to enhance the bactericidal activity of phagocytes in dairy cows [2,3]. Changes of multiple inflammatory mediators in milk in response to intramammary (i.mam.) *E. coli* infection have been previously described [4]. Also antimicrobial defence proteins like lactoferrin (Lf) and lysozyme (Lz) are known to increase during clinically apparent mastitis [5,6]. Contrary to Lf, synthesis of the major milk proteins is diminished during inflammation [7,8]. The aim of this study was to determine short-term changes of mRNA expression of various soluble immunologically important factors as well as major milk proteins in mammary tissue in response to LPS-induced mastitis. Therefore, we have developed a new biopsy method to obtain repeated tissue samples during a 12-h period.

2. Materials and methods

2.1. Cows

Five healthy dairy cows (four German Simmental, one Brown Swiss) in their first to fourth lactation, free of intramammary inflammation, and milking an average of 19 kg per day were used for this experiment. To allow adaptation to the new environment, the animals were transferred to the experimental stable 7 days before the experiment. Quarter milk samples were collected daily for 7 days before inoculation for bacteriological examination and determination of milk somatic cell count (SCC). Only quarters with a SCC $<$150,000 cells/ml and milk samples that cultured negative for mastitic pathogens were accepted for the study. To ensure this SCC level until the start of the experiment, the quarters were carefully milked out after oxytocin injection (30 IU, i.m.) at each milking.

2.2. Treatments

On the day of experiment cows were intramammarily (i.mam.) injected in one quarter (LPS quarter) with 100 μg of *E. coli*-lipopolysaccharide (Serotype O26:B6; Sigma Chem. Co., St. Louis, USA) in 10 ml saline and the contralateral quarter (control quarter) with 10 ml saline (9 g/l) through the teat canal immediately after morning milking. Shortly before injection the mammary glands were cleaned and the teats were disinfected. Mammary biopsy samples of both quarters were taken immediately before (0 h) and at 3, 6, 9 and 12 h after inoculation, snap frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until used for mRNA analyses. Blood samples used for haematological studies were taken following the

same time course and their coagulation was prevented by EDTA. Rectal temperature was measured at 1-h intervals until 12 h after LPS challenge.

2.3. Biopsy procedure

On the day of experiment the two experimental quarters, either the right and left rear gland or the right and left front gland, were clipped and cleaned. After morning milking and immobilisation of the cows, biopsy sampling was started while cows remained standing and did not receive sedation. Biopsy samples from the rear quarters were taken from caudal direction and those from the front glands were taken laterally. The biopsy site which was carefully selected to avoid the cisternal region and larger subcutaneous blood vessels, was washed and sterilized with 70% ethanol. Thereafter, the skin of this area was first anaesthetized with Xylocain pump spray (AstraZeneca, Wedel, Germany) before anaesthetizing the biopsy site by subcutaneous injection of 1 ml lidocain (Chassot, Ravensburg, Germany). A 1–1.5-cm incision was made through the skin and the connective tissue capsule, using shears and tweezers, and the secretory tissue was exposed. Afterwards two biopsies per quarter were carried out through this incision, using a human Bard®Magnum®Biopsie instrument (BARD, Covington, GA) and a Core Tissue Biopsy Needle (12 g × 10 cm) (BARD), and two cores of mammary tissue (30–60 mg) were extracted. To control bleeding caused by the biopsy a swab was pressed into the biopsy area. Until the second biopsy of this quarter 3 h later, carried out through the same incision, the biopsy site was only packed with a swab and Fixomull®stretch (Beiersdorf AG, Hamburg, Germany) avoiding any contamination. Subsequently the same procedure was repeated with the contralateral quarter. For additional mammary biopsy samples which were taken from the same area at 3, 6, 9 and 12 h after induction of mastitis the entrance angle of the needle was slightly changed in order to obtain tissue from different regions at each sampling. Immediately after the experiment and for 3 days following the biopsy sampling all animals received antibiotic prophylaxis as well as anti-inflammatory therapy and the wound dressing was changed every day until the biopsy site was healed. During this period cows were not milked.

2.4. Determination of blood leukocytes

Blood total leukocyte number was measured with an automatic animal blood counter (Scil Animal Care Company GmbH, Viernheim, Germany).

2.5. Total RNA extraction and cDNA synthesis

Total RNA of mammary biopsy samples was isolated using TriPure (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations, and tissue homogenization was implemented by Dstroy-Sticks (Hessisch Oldendorf, Germany). To quantify the amount of total RNA extracted, optical density (OD) was determined at three different dilutions of the final RNA preparations at 260 nm, corrected by the 320 nm background absorption. RNA integrity was verified by the OD_{260}/OD_{280} absorption ratio> 1.8. Synthesis of first strand complementary DNA (cDNA) was performed with reverse transcriptase (MMLV-RT, Promega, Madison, WI, USA) and random hexamer primers (MBI

Fermentas, St. Leon-Rot, Germany) according to the manufacturers instructions. Final concentration of reversely transcribed total RNA was 25 ng/μl.

2.6. Oligonucleotide primers

Primer pairs were either newly designed using published bovine or human nucleic acid sequences or previously published primer sequences were used [9–12]. To demonstrate the amplification of only the cDNA but not the genomic DNA the sense and antisense primers of each target gene were designed to produce an amplification product which spanned at least two exons in the highly conserved coding region (CDS) of the appropriate coding sequence of multiple species. Primer design and optimization were carried out in the high homology regions of the multiple alignment with regard to primer dimer formation, self-priming formation and primer melting temperature (HUSAR program, DKFZ, Heidelberg, Germany). Primer informations are listed in Table 1.

2.7. Quantification by real-time PCR

Polymerase chain reaction was performed in the LightCycler (Roche Diagnostics) with 25 ng and accordingly 10 ng (for milk proteins) reversely transcribed total RNA. Further reaction components for the LightCycler reactions were 1.0 μl LightCycler DNA Master SYBR Green I (Roche Diagnostics), 1.2 μl $MgCl_2$ (4 mM), 0.2 μl forward primer (0.4 μM), 0.2 μl reverse primer (0.4 μM) and water up to a final volume of 10 μl. Prior to amplification an initial denaturation step was performed ensuring complete denaturation of cDNA. All PCR reactions were performed with 40 cycles, depending on factors tested, and product specific PCR cycle conditions are listed in Table 2. To each amplification cycle a fourth segment with an elevated temperature fluorescence acquisition point was added to remove unspecific signals before SYBR Green I quantification, permitting a more specific quantification of PCR products. After the last amplification cycle, PCR products were specified in a melting curve analysis as each product has its specific melting temperature.

To verify the specificity of each PCR quantification real-time amplified RT–PCR products were sequenced and a nucleotide sequence identity to the published bovine sequence of at least 98–100% could be confirmed (HUSAR program, DKFZ, Heidelberg, Germany).

In order to confirm a constant housekeeping gene (hkg) expression level in the investigated total RNA extractions derived from the different samples, additionally a ubiquitin (UbC) RT–PCR was performed.

2.8. Mathematical and statistical evaluations

A relative quantification according to UbC was performed. For the determination of expression levels only the crossing point (CP) values obtained by the LightCycler software (3.1) based on the "*Fit Point Method*" fluorescence acquisition were calculated. Analysis line was set to a constant fluorescence level of 1 where measurement of CPs was done. In the "*Fit Point Method*" the threshold fluorescence level and therefore the amount of PCR products was identical for all samples. Expression data were calculated according to

Table 1
Sequence of PCR primers (forward: for; reverse: rev), PCR product length and GenBank and EMBL accession number (species in parentheses) of the used published nucleic acid sequences

Primer	Sequence (5′ → 3′)	Length (bp)	EMBL accession number
UbC for UbC rev	AGA TCC AGG ATA AGG AAG GCA T GCT CCA CCT CCA GGG TGA T	198	Z18245 (bovine)
TNF-α for TNF-α rev	TAA CAA GCC GGT AGC CCA CG GCA AGG GCT CTT GAT GGC AGA	277	AF011926 (bovine)
IGF-I for IGF-I rev	TCG CAT CTC TTC TAT CTG GCC CTG T GCA GTA CAT CTC CAG CCT CCT CAG A	240	X15726 (bovine)
PAF-AH for PAF-AH rev	CGA TTT ATT CTG CTA TTG GCA TTG GGC TCC ACC AAA AGA ATG TCC	362	U34247 (bovine)
COX-2 for COX-2 rev	TCT TCC TCC TGT GCC TGA T CTG AGT ATC TTT GAC TGT GG	358	AF031698 (bovine)
5-LO for 5-LO rev	GCC CTT CTA CAA CGA CTT CGA CAG GGT TCC ACT CCA TCC A	332	AJ306424 (bovine)
Lf for Lf rev	GGCCTTTGCCTTGGAATGTATC ATTTAGCCACAGCTCCCTGGAG	338	L08604 (bovine)
Lz for Lz rev	GAG ACC AAA GCA CTG ATT ATG GGA TCC ATG CCA CCC ATG CTC TAA	195	U25810 (bovine)
iNOS for iNOS rev	ACC TAC CAG CTG ACG GGA GAT TGG CAG GGT CCC CTC TGA TG	316	U14640 (bovine) L09210 (human)
αS1-CN for αS1-CN rev	GAA CTG AGC AAG GAT ATT GGG A TAG GCA TCC AGC TGG TAG AAT	362	M33123 (bovine)
αS2-CN for αS2-CN rev	GGA CGA TAA GCA CTA CCA GA TGG CTT CAT AGC TTT CTG ATG C	358	M16644 (bovine)
β-CN for β-CN rev	TCC CTA AAT ATC CAG TTG AGC C TCC TGG TAC AGC AGA AAG GC	253	M16645 (bovine)
κ-CN for κ-CN rev	ACC AAC AGA AAC CAG TTG CAC CTA CAG TGC TCT CTA CTG CTT	303	M36641 (bovine)
α-LA for α-LA rev	ACC AGT GGT TAT GAC ACA CAA GC AGT GCT TTA TGG GCC AAC CAG T	233	M18780 (bovine)
b-LG for b-LG rev	AGA TCG ATG CCT TGA ACG AGA A TGT CGA ATT TCT CCA GGG CCT	165	X14712 (bovine)

the equation:

$$\Delta \mathrm{CP}_{\mathrm{control}[X]} = \mathrm{mean}\,\mathrm{CP}_{\mathrm{control}[0]} - \mathrm{mean}\,\mathrm{CP}_{\mathrm{control}[X]}$$

$$\Delta \mathrm{CP}_{\mathrm{treatment}[X]} = \mathrm{mean}\,\mathrm{CP}_{\mathrm{treatment}[0]} - \mathrm{mean}\,\mathrm{CP}_{\mathrm{treatment}[X]}$$

$$\text{LPS challenge for } X \text{ hours; } [X] = 0, 3, 6, 9 \text{ or } 12\,\mathrm{h}$$

In order to normalize the time-course of biopsy sampling CPs of all samples were substracted from CP value determined for 0 h biopsy, thus leading to a relative expression of $1 = 2^0$ for

Table 2
Product specific Lightcycler PCR conditions

Factor	Denaturation		Primer annealing		Elongation		Fluorescence acquisition	
	°C	s	°C	s	°C	s	°C	s
TNF-α	95	5	62	10	72	20	86	3
IGF-I	95	5	62	10	72	20	86	3
PAF-AH	95	5	62	10	72	20	83	3
COX-2	95	5	62	10	72	20	82	3
5-LO	95	5	62	10	72	20	84	3
Lf	95	5	62	10	72	20	86	3
Lz	95	5	62	10	72	20	84	3
iNOS	95	5	62	10	72	20	88	3
αS1-CN	95	5	62	10	72	20	81	3
αS2-CN	95	5	62	10	72	20	80	3
β-CN	95	5	64	10	72	20	85	3
κ-CN	95	5	62	10	72	20	81	3
α-LA	95	5	62	10	72	20	78	3
b-LG	95	5	62	10	72	20	83	3
UbC	95	5	62	10	72	20	85	3

Abbreviations: TNF-α, tumor necrosis factor-α; IGF-I, insulin-like growth factor-I; PAF-AH, platelet-activating factor acetylhydrolase; COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase; Lf, lactoferrin; Lz, lysozyme; iNOS, inducible nitric oxide synthase; CN, casein; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; UbC, ubiquitin.

respective time-point. Relative expression levels within each factor are given by arithmetic formula $2^{\Delta CP}$. The logarithm dualis ($\log_2$) is based on an optimum efficiency (E) of PCR which is $E = 2$ where the PCR product is completely replicated every cycle. Data are presented as mean ± S.E.M. For statistical analysis, the MIXED procedure of the SAS program (version 8.01) [13] was used. The quarter was the repeated subject. Differences were considered significant if $P < 0.05$.

Example given for iNOS (Fig. 2(d)):

$\Delta CP = 4$ at 3 h means an up-regulation of expression of 2^4 as compared to control expression at time point 0 h.

3. Results

3.1. Rectal temperature and blood leukocytes

Rectal temperature (RT) transiently increased ($P < 0.05$) from 38.7 ± 0.2 °C to peak values of 39.4 ± 0.2 °C at 5 h. RT remained above pre-experimental level through 9 h post-inoculation and then returned to pre-infusion level within 24 h after LPS challenge (Fig. 1).

Blood leukocyte number decreased from $7.7 \pm 1.1 \times 10^9$ l^{-1} at 0 to $5.7 \pm 1.0 \times 10^9$ l^{-1} at 3 h, remaining in the reference range (4.0–10.0) [14], and thereafter recovered to pre-treatment levels until 12 h after LPS administration (Fig. 1).

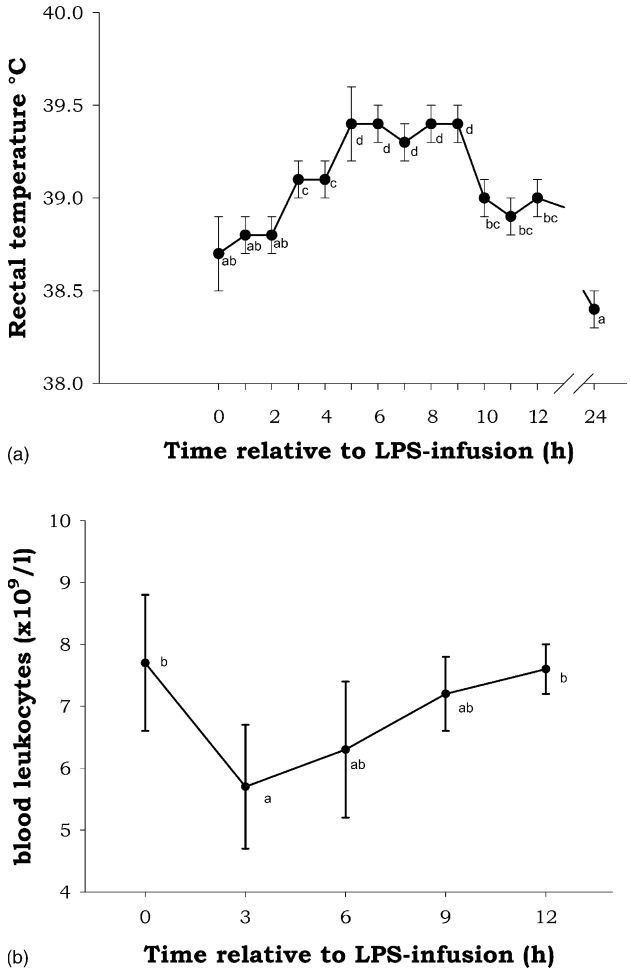

Fig. 1. Rectal temperature (a) and circulating leukocytes (b) before and after intramammary injection of *E. coli* endotoxin. Data are means ± S.E.M. of five cows. Means without common superscripts (a, b, c and d) are significantly different ($P < 0.05$).

3.2. Cytokines

Tumor necrosis factor-α (TNF-α) in LPS-challenged quarters increased ($P < 0.05$) to highest values at 3 h after LPS challenge, about 47-fold higher than baseline levels. Thereafter the following times of measurement showed a clear decrease. A significant change was also seen in control quarter but less than in the LPS quarter. Significant differences of TNF-α-mRNA expression between LPS- and control quarter were observed at 3 and 6 h post-inoculation (LPS > control) (Fig. 2).

Insulin-like growth factor-I (IGF-I)-mRNA expression did not change significantly (Table 3).

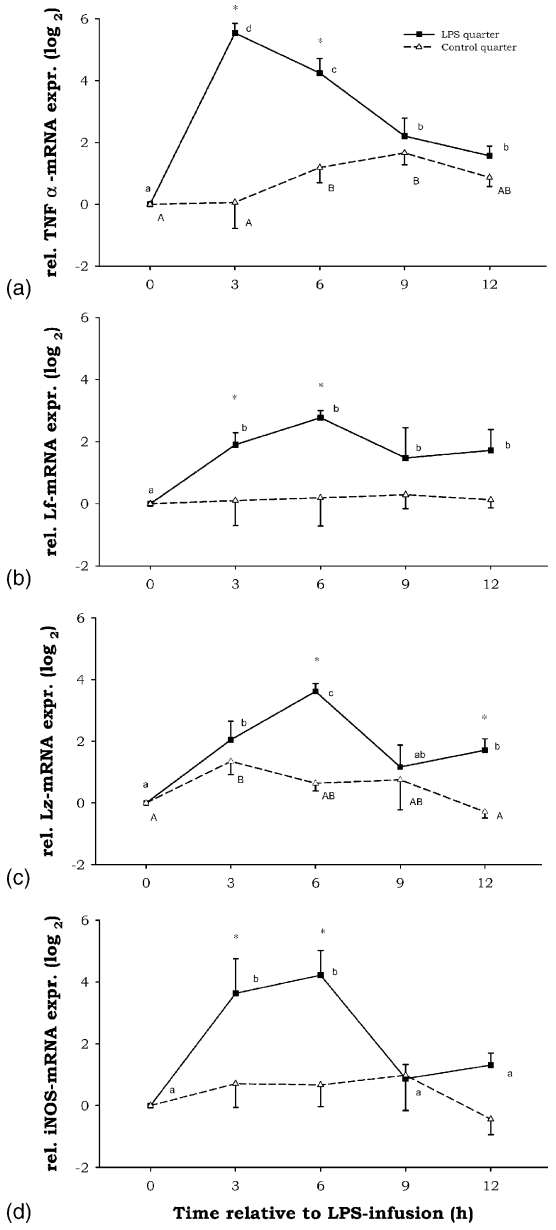

Fig. 2. Relative mRNA expression of tumor necrosis factor-α (TNF-α) (a), lactoferrin (Lf) (b), lysozyme (Lz) (c) and inducible nitric oxide synthase (iNOS) (d) in mammary biopsy samples of quarters intramammarily infused with LPS (LPS quarter) or with saline (control quarter). Data are means ± S.E.M. of five cows. Means within LPS quarter without common superscripts (a, b, c and d) and means within control quarter without common superscripts (A and B) are significantly different ($P < 0.05$). * $P < 0.05$, means of LPS and control quarters are significantly different at a given time.

Table 3
Relative mRNA expression of inflammatory factors and housekeeping gene (hkg) ubiquitin (UbC) relative to intramammary injection of *E. coli* endotoxin (LPS)

	Time after endotoxin-infusion							
	3 h		6 h		9 h		12 h	
	Control (means ± S.E.M.)	LPS (means ± S.E.M.)	Control (means ± S.E.M.)	LPS (means ± S.E.M.)	Control (means ± S.E.M.)	LPS (means ± S.E.M.)	Control (means ± S.E.M.)	LPS (means ± S.E.M.)
Changing of mRNA expression, $\log_2$ (relative to time 0)								
COX-2	1.51 ± 1.05	4.45[a,c] ± 0.89	1.23 ± 0.54	3.75[a,c] ± 0.63	2.81 ± 1.62	−0.76[c] ± 1.25	−0.83 ± 0.30	0.07 ± 0.43
PAF-AH	0.29 ± 0.82	0.82 ± 0.47	0.29 ± 1.01	1.45 ± 0.41	0.12 ± 0.80	−0.23 ± 0.44	−0.63 ± 0.68	0.50 ± 0.83
IGF-I	0.59 ± 0.96	0.02 ± 0.45	1.42 ± 1.20	0.92 ± 0.81	1.48 ± 1.17	0.34 ± 0.67	0.60 ± 0.52	0.50 ± 0.45
5-LO	−0.54[b] ± 0.48	−0.62 ± 0.48	−0.37[b] ± 0.33	−1.02[a] ± 0.44	−0.66[b] ± 0.36	−2.15[a,c] ± 0.59	−1.11[b] ± 0.36	−1.26[c] ± 0.13
UbC	0.08 ± 0.33	0.18 ± 0.47	0.50 ± 0.33	0.36 ± 0.43	0.41 ± 0.34	−0.06 ± 0.37	−0.40 ± 0.13	−0.24 ± 0.19

[a] Means of LPS-treated quarters differ significantly ($P < 0.05$) from 0 h.
[b] Means of control quarters differ significantly ($P < 0.05$) from 0 h.
[c] Means of LPS and control quarters are significantly different ($P < 0.05$).

Table 4
Relative mRNA expression of milk proteins relative to intramammary injection of *E. coli* endotoxin (LPS)

	Time after endotoxin-infusion							
	3 h		6 h		9 h		12 h	
	Control (means ± S.E.M.)	LPS (means ± S.E.M.)	Control (means ± S.E.M.)	LPS (means ± S.E.M.)	Control (means ± S.E.M.)	LPS (means ± S.E.M.)	Control (means ± S.E.M.)	LPS (means ± S.E.M.)
Changing of mRNA expression, $\log_2$ (relative to time 0)								
αS1-CN	−0.58 ± 0.69	−0.33 ± 0.44	−1.20 ± 0.81	0.09 ± 1.00	−1.59 ± 0.70	−0.88 ± 0.25	−0.80 ± 0.35	−0.45 ± 1.08
αS2-CN	−0.61 ± 0.65	−0.50 ± 0.35	−1.40 ± 0.91	−0.27 ± 1.05	−1.63 ± 0.82	−1.46 ± 0.11	−1.07 ± 0.29	−0.97 ± 1.04
β-CN	−0.83 ± 0.74	−0.75 ± 0.34	−1.29 ± 1.02	−0.51 ± 1.25	−1.74 ± 0.81	−1.99 ± 0.48	−1.39 ± 0.26	−1.32 ± 1.17
κ-CN	−0.33 ± 0.36	0.34 ± 0.46	−0.60 ± 0.63	−0.04 ± 0.45	−1.09 ± 0.55	−1.83[a] ± 0.60	−0.65 ± 0.24	−0.67 ± 0.64
α-LA	−0.96 ± 0.89	−1.42 ± 0.58	−2.26 ± 1.21	−1.38 ± 1.73	−3.14[b] ± 1.44	−2.50[a] ± 0.36	−2.14 ± 0.32	−2.13 ± 1.49
b-LG	−0.11 ± 1.09	−0.23 ± 0.39	−1.48 ± 1.32	0.43 ± 1.42	−1.47 ± 1.31	−1.34 ± 0.22	−0.67 ± 0.53	−0.40 ± 1.71

[a] Means of LPS-treated quarters differ significantly ($P < 0.05$) from 0 h.
[b] Means of control quarters differ significantly ($P < 0.05$) from 0 h.

3.3. Lipidmediators (key enzymes of their metabolism)

Similar to TNF-α the cyclooxygenase-2 (COX-2)-mRNA expression increased ($P < 0.05$) to its highest levels within 3 h in LPS-treated quarters and mRNA expression in LPS- and control quarters were also significantly different at 3, 6 and additionally 9 h after LPS administration (Table 3).

Platelet-activating factor acetylhydrolase (PAF-AH)-mRNA expression also tended to increase until 6 h post-inoculation as compared to pre-experimental level ($P = 0.07$) (Table 3). Contrary to these two lipidmediators, 5-lipoxygenase (5-LO) showed a decrease ($P < 0.05$) in mRNA expression in LPS as well as in control quarter, whereas significant differences between LPS- and control quarter were seen 9 h after LPS inoculation (Table 3).

3.4. Antimicrobial proteins and enzymes

mRNA expression of lactoferrin (Lf), lysozyme (Lz) and inducible nitric oxide synthase (iNOS) increased ($P < 0.05$) within 3 h after LPS injection and peaked at 6 h after challenge. Observed baseline values for example of iNOS-mRNA expression were up to 19-fold lower than maximal expression reached in udder tissue. For Lz, a significant, albeit in comparison to the LPS quarter small rise of mRNA expression, was seen in the control quarter. In relation to the control quarter mRNA expressions in the LPS quarter are significantly increased for Lf and iNOS 3 and 6 h and for Lz 6 and 12 h after LPS mastitis induction, respectively (Fig. 2).

3.5. Milk proteins

αS1-Casein (CN)-, αS2-CN-, β-CN- and β-lactoglobulin (β-LG)-mRNA expression did not change significantly but values were tendentially ($P = 0.26, 0.09, 0.05, 0.24$, respectively) lower at 9 h after LPS administration as compared to 0 h (Table 4). Decreasing ($P < 0.05$) in mRNA expression relative to time 0 at 9 h post-inoculation was also observed for α-lactalbumin (α-LA) in both quarters and for κ-CN only in the LPS quarter (Table 4).

3.6. Housekeeping gene

Changes of ubiquitin (UbC)-mRNA expression were tendentially smaller than those observed for other mRNA and did not approach statistical significance (Table 3).

4. Discussion

During mammary infection, non-specific responses are the predominant defences. For our investigation, eight soluble immunologically important factors were selected, which are known to be involved in the natural defence mechanisms of the mammary gland against invading microorganisms and which were expected to change within 12 h from infection. Simultaneously the mRNA expression of the major six milk proteins in udder tissue were investigated. For the most part of investigated factors was already known that their concentration

in milk changes on protein level during mastitis. Our own study indicated these changes occurring also in mRNA expression.

Due to our newly developed biopsy technique which was rapid and could be used in awake and standing animals, not sedated cows we were able to perform serial samplings. Physiological changes could be investigated in the same quarter and therefore conclusive results about changes of mRNA expression during the acute phase of inflammation were retrieved. It could be expected that the surgical procedure of taking biopsies itself represents an irritation which leads to "slight inflammatory reactions". Interestingly, for some factors such as TNF-α and Lz a significant rise in mRNA expression was seen in LPS treated as well as in control quarter. This indicates a minor reaction already by the biopsy procedure. Whereas other factors such as iNOS and Lf increased only in the LPS quarter, appearing to need stronger inflammatory stimuli.

The results represented that most of the examined immunologically relevant factors showed significantly different expression levels in the control and LPS quarter, and very distinct for TNF-α, Lf, Lz and iNOS shown in Fig. 2. Changes in ubiquitin mRNA expression levels, serving as housekeeping gene, were not present. Thus, the changes of mRNAs encoding for a variety of immunologically important factors represented specific responses of the mammary gland to LPS challenge.

TNF-α is an important cytokine and neutrophil-attracting factor, therefore playing a major role in the defence against mastitis [21]. The early and high pronounced increase of TNF-α mRNA expression peaking already 3 h after challenge confirms previous studies [15,16]. The impact of TNF-α as one of the cytokines mediating the acute phase response was also demonstrated in this study since increase of rectal temperature and decrease of blood leukocyte number occurred concomitantly with the rise of TNF-α mRNA expression whereas the rectal temperature peak was seen 2 h after the peak of mRNA expression. In a previous study the milk cells were identified as a major source of TNF-α compared to mammary gland tissue [17]. But also in udder tissue TNF-α mRNA expression was found particularly in quarters with sub-clinical mastitis. Rise of TNF-α mRNA expression in LPS challenged quarters as well as in quarters with sub-clinical somatic cell count levels identifies also mammary tissue as an obvious source of TNF-α during inflammation.

Comparable to the TNF-α expression pattern, a rise of Lactoferrin (Lf) mRNA expression was detected. Lf expression was significant up-regulated in LPS-treated quarters and not in control quarters. Lf is known to increase in bovine milk during clinical mastitis [5,18]. Increased concentration of Lf in the mammary secretion during mastitis or involution indicates that the regulation of Lf in the mammary gland is contrary to that of other milk proteins [8]. These changes were paralleled by changes in Lf mRNA expression [19]. Herein the increase was already seen 3 h after LPS application with the highest value at 6 h. Although Lf is released from neutrophils during inflammation [20], the elevation of Lf mRNA expression in udder tissue seen in this study gave additional support to the theory that Lf in udder secretions originates also in the secretory epithelium, suggested already in studies [21,17].

Also in this study a significant increase of Lz mRNA expression was obvious with peak values at 6 h after LPS inoculation. Lz is of relevance to the natural defence system of the mammary gland due to its bacteriostatic and even bactericidal effects on udder pathogens [22]. In several earlier studies was shown that concentration of Lz is low in normal bovine

milk but increases during mastitis [6,21]. Significant correlation between concentrations of Lz and SCC have also been reported [21,23]. Persson et al. [21] and Steinhoff et al. [24] concluded that leukocytes are the most likely source of Lz during inflammation but a role of the mammary epithelial cells seems likely.

Intra-mammary infusion of LPS caused an enhanced i.mam. production of NO [15,25]. In accordance LPS challenge augmented iNOS mRNA expression in this study. Activated macrophages synthesize nitric oxide (NO) to eliminate intracellular pathogens. Therefore NO has a key role in mediating microbistatic and/or microbicidal activity [26]. NO synthesis is catalysed by inducible nitric oxide synthase (iNOS) which is known to be induced by LPS.

TNF-α is supposedly responsible for increased NO production [15]. This assumption is supported by our expression data, because the peak of TNF-α mRNA expression preceded the peak of iNOS-mRNA expression.

Possibly iNOS or rather NO was responsible for a suppression of 5-lipoxygenase (LO) mRNA expression seen in this study. NO is assumed to inhibit the activity of 5-lipoxygenase [27]. Lipid mediators such as prostaglandin's (PGs) and leukotrienes, i.e. eicosanoids, are derived from arachidonic acid (AA) and are among the most important mediators and modulators of the inflammatory reaction [28]. To determine synthesis of PGs and leukotrienes the mRNA expression of specific key enzymes of their synthetic pathways, i.e. cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO) was estimated. An increased synthesis of prostanoids (prostaglandins, prostacyclin and thromboxanes) during mastitis was shown based on the rise of COX-2 mRNA expression. Similar to TNF-α, the infusion of endotoxin resulted in a marked increase in COX-2, 3 h after inoculation. In contrast to elevated COX-2 mRNA expression, 5-LO showed a decrease, consistent with earlier studies [12,17], reporting that most likely milk cells are the major source of 5-LO and, as a result, for leukotrienes in the udder. Whether iNOS or NO respectively, known to inhibit the activity of 5-LO [27], was responsible for decreasing in 5-LO-mRNA expression remains questionable.

Further cytokines, like soluble IGF-I in milk have been shown to increase already before SCC influx (3–4 h) during mastitis induced by *E. coli* infection or by *E. coli* endotoxin [29,30]. Evidence also exists for a paracrine or autocrine role of IGF-I because its mRNA was detected in bovine mammary tissue [31]. Beside this IGF-I exerts an endocrine effect on the mammary gland [32]. Surprisingly, in this study neither saline nor endotoxin induced any significant changes in IGF-I mRNA expression levels in mammary tissue. This result questions mammary epithelial and stroma cells as a source of milk IGF-I and points to macrophages and lymphocytes being responsible for the synthesis. Since IGF-I seems to be important for tissue repair and recovery processes because of functioning as a potent mitogen for mammary epithelial cells [33], the absence of changes in IGF-I mRNA expression in this study could also be due to the too weak inflammatory reaction.

The platelet-activating factor (PAF) is a potent pro-inflammatory phospholipid [34]. A key mechanism for the removal of PAF is hydrolysis catalysed by PAF-acetylhydrolase (AH), which converts PAF to the biologically inactive lyso-PAF [35]. Since PAF is a phospholipid it could only be estimated via the mRNA expression of a specific key enzyme of its degradation, PAF-AH, which converts PAF to the biologically inactive lyso-PAF and is highly specific. Furthermore, Cao et al. [34] showed that PAF stimulates the expression of

its own inactivating enzyme, i.e. substrate and PAF-AH are positively correlated. Therefore, elevated PAF-AH expression allows the assumption of an increased PAF synthesis. In this study, PAF-AH mRNA expression in LPS challenged mammary tissue increased only numerically until 6 h after LPS application. Possibly PAF is not a central importance in the short-term response to LPS in the mammary gland.

Mastitis caused changes of milk composition are supposed to be primarily a result of impaired synthetic and secretory activity of the udder epithelial cells as well as a change in the permeability of the milk–blood barrier. Milk of dairy cows secreted in healthy udders has a protein content of about 3–3.5%. The major milk proteins (α-casein, β-casein, α-lactalbumin and β-lactoglobulin) have been reported to decrease while other proteins originating from the blood (serum albumin and immunoglobulins) increase [36].

In this study, a decrease of mRNA expression of α-lactalbumin was evident, which is a co-factor in the lactose biosynthesis. Also TNF-α is hypothesized to be implicated in changes of milk protein composition during mastitis based on changes of milk protein secretion and integrity of the milk–blood barrier [37]. Some of the changes in milk protein composition even appear to be advantageous in the defence system of lactating mammary tissue against infections. Both caseins inhibit myeloperoxidase-mediated oxygen-dependent bactericidal activity of neutrophils [38]. Reduced casein secretion may therefore cause increased activity of this enzyme.

5. Conclusion

In conclusion, this study shows that the employed biopsy technique allows repeated sampling and is suitable to study changes of mRNA expression of various factors during a 12-h period. The mRNA expression levels of most immunologically relevant factors changed within 6 h, showing highest mRNA expression at 3–6 h post-inoculation. Most milk proteins mRNA expression levels of did not change significantly within 12 h after LPS challenge.

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