Haptoglobin Concentrations in Blood and Milk After Endotoxin Challenge and Quantification of Mammary Hp mRNA Expression

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ABSTRACT

Haptoglobin (Hp), an acute phase protein mostly secreted by the liver, is an inflammatory marker. To use the full diagnostic potential of Hp measurements for mastitis, we developed and validated an ELISA sensitive to quantify even basal and subclinical concentrations in both blood and milk. Bovine Hp was purified from serum and was used as a standard and to generate polyclonal antiserum. The limit of detection was 0.07 µg of Hp/mL. From 6 cows challenged by intracisternal injection of lipopolysaccharide (LPS) into one quarter, blood samples were collected 0, 3, 6, 9, and 12 h after LPS administration. Milk samples from the treated and from the contralateral quarters were collected 0, 3, 6, 9, 12, 24, 36, 48, and 60 h after LPS administration. Haptoglobin concentrations in blood were increased above basal at 9 h, whereas milk Hp concentration increased 3 h after LPS administration. We therefore evaluated Hp mRNA synthesis within the mammary gland and specifically demonstrated Hp mRNA expression in parenchymal tissue, in tissue around the cisternal milk ducts and also in teat tissue by RT-PCR. Haptoglobin mRNA expression was then quantitatively evaluated by real-time RT-PCR in mammary biopsies collected from the treated and the control quarter before, and 3, 6, 9, and 12 h after LPS challenge from 6 other cows. Haptoglobin mRNA expression in the treated vs. the control quarters was different. The relation between mammary Hp expression and milk Hp concentrations needs further investigation, but the results suggest good diagnostic potential of this parameter for mastitis.

(Key words: mammary gland, haptoglobin, mastitis, dairy cow)

Abbreviation key: APP = acute phase protein, bHp = bovine haptoglobin, CRP = C-reactive protein, Hp = haptoglobin, OD = optical density.

INTRODUCTION

Acute phase proteins (APP) are a group of serum proteins that undergo substantial quantitative changes in response to infection, inflammation, or trauma (Heinrich et al., 1990; Eckersall, 1995). Stimulated by interleukin-1, interleukin-6, and tumor necrosis factor alpha, hepatocytes increase protein synthesis and secretion. Many of the APP operate in host defense. They comprise complement components, clotting factors, protease inhibitors, and metal binding proteins. Besides those APP that may increase a thousand-fold following bacterial invasion and tissue damage, e.g., C-reactive protein (CRP) in humans, there is a second group of APP where concentrations increase 2- to 3-fold, and haptoglobin (Hp) belongs to this latter group. The changes occurring are species-specific: In cattle, CRP is not an APP even though it is found in normal serum (Kent, 1992). In contrast, Hp is a major APP in ruminants and has been suggested as a diagnostic marker for mastitis (Eckersall et al., 2001) and respiratory diseases (Heegaard et al., 2000) in cattle. Serum concentrations of Hp reportedly increase in dairy cows with naturally occurring clinical mastitis and in cows in which mastitis has been induced experimentally by intramammary bacterial challenge (Conner and Eckersall, 1986; Hirvonen et al., 1996; Salonen et al., 1996). For the determination of Hp in blood, both biochemical and immunological assays are available and have successfully been used to characterize Hp serum concentrations in cattle. The hypothesis that Hp as a major APP in cows might be transferred into milk during the acute phase response caused by mastitis has been substantiated by using an immunodiffusion assay in milk from cows affected with mastitis (Eckersall et al., 2001). Biochemical assays are not applicable to milk samples because the milk peroxidase activity interferes with the assay, i.e., quantification of peroxidase activity of hemoglobin preserved by complexing with Hp (Eckersall et al., 2001). Pedersen et al. (2003) used a sandwich ELISA to measure Hp in blood and milk after intramammary challenge with *Streptococcus uberis*. Both methods (the immunodiffusion assay and the sandwich ELISA) were

Received September 23, 2003.

Accepted June 8, 2004.

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not sufficiently sensitive to determine Hp concentrations in milk samples from healthy cows.

The aim of this study was to develop an ELISA that is sufficiently sensitive to determine Hp concentrations in both serum and milk under basal conditions as well as during inflammation. This test was applied to milk and serum samples from cows following experimentally induced sterile mastitis. The results led to the hypothesis that Hp might not only be transferred from blood into milk but could also be locally synthesized within the mammary gland. For this reason, we evaluated whether Hp mRNA is detectable in mammary gland tissue extracts and whether LPS-induced alterations of the expressions might be observed.

MATERIALS AND METHODS

Development of an ELISA for Bovine Haptoglobin (bHp)

Purification of bHp. Acute phase sera were obtained from cows with clinically severe mastitis or metritis. bHp was purified from these sera by affinity chromatography on hemoglobin-sepharose followed by gel filtration, as described previously for porcine Hp (Hiss et al., 2003). The purity of the Hp preparation obtained was assessed by SDS-PAGE performed according to Laemmli (1970) using a 5.6% stacking gel and a 12% resolving gel of acrylamide. Precision Protein Standards (Bio-Rad, Hercules, CA) were used as molecular weight markers. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R 250 (Roth, Karlsruhe, Germany).

Generation of polyclonal antibodies against bHp. An amount of 200 μ g of purified bHp was emulsified in Freund's complete adjuvant (Sigma Aldrich, Taufkirchen, Germany) and injected subcutaneously into rabbits at multiple sites. For subsequent immunizations, 50 or 100 μ g of purified bHp in Freund's incomplete adjuvant (Sigma Aldrich) were used. One week after each booster injection, the rabbits were bled from an ear vein. Blood was allowed to clot at room temperature, and after centrifugation ($2000 \times g$), aliquots of antiserum were stored at -20°C . Titers and binding specificity were tested by ELISA and Western blotting as described below, and the antiserum with the highest titer was selected for use.

Western immunoblotting. Electrophoresis was performed as described above. In addition to the bHp preparation, bovine and caprine serum from clinically healthy animals and also serum from a cow with acute mastitis were used in SDS-PAGE. Acrylamide gels from electrophoresis were electro-blotted onto PVDF membranes (Hybond-P, Amersham Biosciences, Freiburg, Germany), and membranes were then blocked with

2.5% casein for 20 min. The rabbit antiHp serum was diluted 1/10,000 in assay buffer (0.12 M NaCl, 0.02 M Na_2HPO_4 , 0.01 M EDTA, 0.1% hydrolyzed gelatin, 0.005% chlorhexidine digluconate 20%, 0.05% Tween 20, 0.002% phenol red, pH 7.4) containing 0.5% casein. After incubation for 1 h, the membrane was washed in assay buffer and incubated with the second antibody conjugated to peroxidase (monoclonal antirabbit IgG, γ -chain specific, Sigma Aldrich) for 30 min (dilution 1/10,000). After 3 washes with washing buffer (10% PBS, pH 7.4, 0.05% Tween 20), the substrate (3,3'-diaminobenzidine-tetrahydrochloride) was added. The reaction was stopped with distilled water.

Development of the bHp ELISA. Microtiter plates (EIA plate 9018, Corning Costar, Cambridge, MA) were coated with purified bHp (5 ng in 100 μ L of 50 mM NaHCO_3 , pH 9.6) at 4°C for 20 h. After blocking with 300 μ L of 2.5% casein in 0.05 M NaCl, pH 7.4, at room temperature for 1.5 h, the plates were stored at -20°C . Prior to use, the plates were washed 5 times. To each well, 50 μ L of test sera (dilution 1/100 in healthy cows or 1/1000 in diseased cows) was added in duplicate. For Hp quantification in milk, skim milk was used accordingly (dilution 1/10 or 1/100). Calibration curves were created using 50 μ L of purified bHp at dilutions from 0.0 to 10 $\mu\text{g/mL}$ in duplicate. An amount of 50 μ L of the antiserum (dilution 1/50,000) was then added and incubated for 2 h at room temperature. After 3 washes, 100 μ L of the second antibody conjugated to peroxidase (1/20,000 dilution) was added and incubated for 30 min. After 5 washes, the wells were filled with 150 μ L of a freshly prepared substrate solution containing 0.05 M citric acid, 0.055 M Na_2HPO_4 , 0.05% urea hydrogen peroxide, 2% ProClin 150, and 2% of a tetramethylbenzidine solution (12.5 mg/mL dimethylsulfoxide). The reaction was stopped after 30 min with 50 μ L of 1 M oxalic acid, and the optical density (OD) was determined at 450 nm with a microtiter plate reader (ELX800, Biotec Instruments, Inc., Winooski, VT). The Hp concentrations in unknown samples were then calculated from the calibration curve.

Twenty-nine serum samples were analyzed for their Hp content by the ELISA developed herein and also by the haptoglobin phase kit (Tridelta Development Ltd., Greystones, Ireland) based on the hemoglobin-binding capacity according to the manufacturer's instructions.

Animals, Treatments, and Sample Collection

To evaluate the presence and distribution of Hp mRNA in the bovine mammary gland, tissue samples were collected from 4 lactating dairy cows at a local slaughter house. Tissue cubes of approximately 0.5 cm^3 in size were dissected from the liver, the teat, from the

cisternal region containing the large milk duct epithelia, and from the glandular parenchyma. Samples for the qualitative analysis were frozen in dry ice.

Endotoxin-induced alterations of milk and mammary Hp mRNA were investigated in 2 animal experiments. Both experiments were approved by the Animal Ethics committee of Bavaria. For experiment one, 6 healthy lactating cows in their first to fourth lactation (4 German Simmental and 2 Brown Swiss) milking an average of 19 kg/d were used. The cows were transferred to the experimental stable and were then allowed to get accustomed to the new environment. Quarter milk samples were collected daily for microbiological testing and for determinations of milk SCC. Only quarters with SCC below 150,000 cells/mL and with milk free of mastitis pathogens were used for the studies. All animals were injected with oxytocin (30 I.U. i.m.) after each machine milking (every 12 h), and the residual milk was subsequently milked out by machine. On the day of the experiment, one quarter per cow was randomly assigned to treatment, and the contralateral quarter was assigned to control. An amount of 100 μ g of *Escherichia coli*-LPS (serotype O26:B6; Sigma Chem. Co., St. Louis, MO) in 10 mL of saline was injected into the teat cistern of the treatment quarter. The control quarter was injected with 10 mL of saline. Mammary biopsies were obtained immediately before (0 h) and 3, 6, 9, and 12 h after the LPS injections, as described in detail by Schmitz et al. (2004). In brief, the skin of the selected biopsy site was disinfected with ethanol (70%), anaesthetized with Xylocain spray (AstraZeneca, Wedel, Germany), and injected subcutaneously with 1 mL of lidocain (Chassot, Ravensburg, Germany). A 1- to 1.5-cm incision was made through the skin and the connective tissue capsule using shears and tweezers, and the secretory tissue was exposed. Biopsies were then taken using a BardMagnum Biopsy instrument (Bard, Covington, GA) with a 12-g $\times$ 10-cm biopsy needle (Bard, Covington, GA). At each sampling time, 2 cores of mammary tissue (30 to 60 mg) were collected through the initial incision, but the entrance angle of the needle was changed to obtain tissue from areas not sampled previously. The biopsy samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until used for mRNA analyses. The incision wound was dressed between the biopsy procedures. Immediately after the experiment, all animals received antibiotic prophylaxis and anti-inflammatory therapy. The wound dressings were changed daily until healing. During the experiment and the 3 d of postexperimental treatments, the cows were not milked.

The biopsy procedure lead to visible alterations of the milk with blood impurities. To obtain normal milk for measurements of the Hp protein, a separate experi-

ment with another 6 cows (6 Brown Swiss, first to fifth lactation) was performed. The experimental setup for experiment 2 was basically the same as for experiment 1, but only quarter milk samples were collected for determinations of SCC and Hp at 0, 3, 6, 9, 12, 24, 48, and 60 h after challenge from the infected and from the control quarters. Besides the milk samples that were taken, cows were milked every 12 h. In addition, blood samples for Hp determination were collected 0, 3, 6, 9, and 12 h after LPS administration from the jugular vein.

Qualitative mRNA Analysis

Total RNA extraction from liver and mammary tissues from slaughtered cows was done according to the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). The total RNA extracted was treated with DNase I, RNase free (Boehringer, Mannheim, Germany) according to the manufacturers' instructions. The enzyme was then heat-inactivated for 5 min at 75°C . Concentration of total RNA in each sample was quantified by absorbance readings at 260 nm, and the integrity of the RNA was checked by ethidium bromide staining after formaldehyde gel electrophoresis. An amount of 1.8 μ g total RNA was reverse transcribed using 200 U reverse transcription (MBI Fermentas, St. Leon Rot, Germany) in reaction buffer (50 mM Tris-HCl, 50 mM KCl, 4 mM MgCl_2 , pH 8.3) with 10 mM DTT, 50 pmol of random hexamer primers (Invitrogen, Karlsruhe, Germany), 500 μ M of dNTPs, and 20 U of ribonuclease inhibitor (MBI Fermentas) in a 20- μ L volume for 10 min at 27°C , 60 min at 42°C , and 1 min at 99°C to inactivate the reverse transcription. Negative controls were performed in the absence of total RNA.

Polymerase chain reaction was done with 3 μ L of cDNA synthesis-product, forward primer 5' GTCTCCCAGCATAACCTCATCTC 3' and reverse primer 5' AACCACCTTCTCCACCTCTACAA 3' (20 pmol of each) spanning the region 33 to 207 bp of the partially published cDNA sequence of bHp (NIH GenBank accession no.: AJ271156), 1 U of Taq DNA-polymerase (MBI Fermentas) in a final volume of 50 μ L with 75 mM Tris-HCl, 2 mM MgCl_2 , 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01% Tween 20, pH 8.8, according to the following temperature-time protocol: 95°C for 60 s, 32 cycles of 94°C for 40 s, 55°C for 20 s, 72°C for 20 s, and final extension at 72°C for 5 min. An amount of 10 μ L of the amplified products was analyzed on a 2% agarose gel by ethidium bromide staining (FluorImager SI, Amersham Pharmacia Biotech, Uppsala, Sweden). The specificity of the resulting 174-bp DNA fragment was verified by direct sequencing of the PCR product (Pharmis, Bonn, Germany).

Quantitative mRNA Analysis

Total RNA of mammary biopsy samples was isolated using TriPure (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. To quantify the amount of total RNA extracted, OD was determined at 3 dilutions of the final RNA preparations at 260 nm. Synthesis of cDNA was performed with reverse transcription (MMLV-RT, Promega, Madison, WI) and random hexamer primers (MBI Fermentas) according to the manufacturer's instructions. Polymerase chain reaction was performed in the LightCycler (Roche Diagnostics, Mannheim, Germany) with 25 ng of reverse transcribed total RNA. Primers were as described above for qualitative analyses. The other components used for the LightCycler reactions were 1.0 μ L of LightCycler DNA Master SYBR Green I (Roche Diagnostics), 1.2 μ L of $MgCl_2$ (4 mM), 0.2 μ L of forward primer (0.4 μ M), 0.2 μ L of reverse primer (0.4 μ M), and water to a final volume of 10 μ L. Prior to the amplification, an initial denaturation step was performed to ensure complete denaturation of cDNA. For the amplification, the same temperature-time protocol as described for the qualitative approach above was used. 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, thus permitting a more specific quantification of PCR products. After the last amplification cycle, the PCR product was specified in a melting curve analysis by its specific melting temperature.

To confirm a constant housekeeping gene expression level in the RNA extracts derived from the different biopsies, ubiquitin expression was analogously quantified by real time-PCR as described earlier (Schmitz et al., 2004).

Mathematical Evaluation of Hp mRNA Expression

A relative quantification normalized to an assumed constant ubiquitin mRNA expression was performed. For the determination of the expression, the crossing point (CP) values obtained by the LightCycler software (3.1) based on the Fit Point method fluorescence acquisition were used for calculations. In each ideal PCR cycle, the number of copies of a cDNA strand doubles. Thus, a change of the crossing point result of 1 means a change of the product by factor 2, i.e., the crossing point represents the logarithm dualis of the amount of cDNA. Calculations were described previously in detail by Schmitz et al. (2004). To eliminate pretreatment variation between samples and to visualize changes with time, the CP results of repeated biopsy samples

Figure 1. (A) SDS-PAGE of purified bovine haptoglobin (bHp): Lane 1: molecular weight marker, Lane 2: purified bHp in the absence of 2-mercaptoethanol, Lane 3: purified bHp in the presence of 2-mercaptoethanol, Lane 4: bovine serum sample. (B) Western blot after reducing SDS-PAGE: Lane 1: purified bHp, Lane 2: goat serum (clinically healthy), Lane 3: bovine serum (clinically healthy), Lane 4: bovine serum (acute mastitis).

were subtracted from the CP value determined for the 0-h biopsy. Data are presented as mean $\pm$ SEM.

Statistical Analysis

All statistical analyses were performed with the SPSS/PC 10.0 program. Nonparametric correlation coefficients (Spearman) were determined to compare Hp concentrations in blood and milk and also to compare the 2 different Hp tests. Changes in Hp concentrations in blood and milk as well as increased mRNA levels compared with baseline levels were tested using the Wilcoxon 2 sample paired signed rank test. Differences in Hp mRNA expression between control quarters and infected quarters were analyzed using repeated measurement analyses (general linear model). Where applicable, Hp concentrations are given as means $\pm$ SEM.

RESULTS

Characterization and Validation of the bHp ELISA

The protocol selected to purify Hp from bovine blood serum yielded a distinct single peak. The purity of this preparation was confirmed by SDS-PAGE (Figure 1A).

Figure 2. Dilutions of purified bovine Haptoglobin (bHp, ◆), blood (▲), and milk (●) showing parallelism between standard curve (bHp), blood serum, and skim milk.

A polyclonal antiserum against the purified bHp was successfully raised, and its specificity was demonstrated by Western blotting (Figure 1B). Positive signals at 34 kDa and at 18 kDa, corresponding to the heavy and light chain, were obtained both from electrophoresed bovine serum (acute mastitis) as well as from the purified Hp preparation. There was no signal in serum from clinically healthy animals (cow and goat).

The enzyme immunoassay developed on the basis of purified bHp and the specific antiserum generated against it was well applicable for bovine samples as demonstrated by parallelism of serial serum and skim milk dilutions with the standard curve (Figure 2). The limit of detection ($0.07 \mu\text{g/mL}$) was estimated as the mean plus 2 standard deviations of 20 zero samples (blanks). Intra- and interassay coefficients of variation were 3.9% ($n = 8$) and 12.16% ($n = 13$), respectively. Compared with the commercial haptoglobin phase kit (Tridelta Development Ltd.), the concentrations measured by ELISA were correlated ($n = 29$, $r = 0.75$, $P < 0.01$).

LPS-Induced Alterations of Hp Concentrations in Blood and in Milk

Injection of LPS into the mammary gland cistern stimulated the cellular defense mechanisms in the treated gland, as indicated by increasing SCC values. At 6 h after LPS injection, 4 of 6 cows showed SCC above the upper limit of detection (10 millions of cells/mL), whereas SCC in control quarters remained unchanged. Lipopolysaccharide treatment also led to an increase of Hp concentrations both in blood serum and in milk obtained from the treated quarter (Table 1). Mean pretreatment values of Hp in blood were 25-fold higher than in milk. In serum, the first significant increase was observed 9 h after injection of LPS ($P <$

0.05). The mean concentrations reached after 12 h were 11.2-fold higher when compared with the initial basal concentration. Haptoglobin concentrations in milk obtained from the quarter used as control remained unchanged. In milk samples obtained from the treated quarter, increased Hp concentrations after 3 h were 35-fold higher than the pretreatment levels ($P < 0.05$). Maximal Hp concentrations of more than 160-fold above basal concentrations were maintained until 12 h after LPS injection.

Hp mRNA Expression in Different Regions of the Mammary Gland

Haptoglobin mRNA transcripts could be demonstrated by RT-PCR in RNA extracts obtained from the liver, the teat, the cisternal region containing the large milk duct epithelia, and also from the glandular parenchyma (Figure 3).

LPS-Induced Alterations of Hp mRNA Expression in Mammary Gland Biopsies

Haptoglobin mRNA expression was influenced both by time ($P < 0.001$) and LPS treatment ($P = 0.003$; time $\times$ treatment $P < 0.03$). As shown in Figure 4, maximal Hp mRNA expression values were reached after 6 h in LPS-treated quarters ($P < 0.05$). The increase in the control quarters was less pronounced, reaching maximum levels after 9 h ($P < 0.05$).

DISCUSSION

The use of APP as markers for the early diagnosis of mastitis is suggested by Eckersall et al. (2001) and Pedersen et al. (2003). In our own studies, increased Hp levels were obvious 3 h after LPS injection, whereas changes in blood occurred later (9 h postinoculation). Pedersen et al. (2003) reported an increase of milk Hp 10 h after experimental induction of mastitis with *Streptococcus uberis*. The authors used a sandwich ELISA for the detection of milk Hp, with a detection limit of $11.5 \mu\text{g/mL}$. However, it is notable that Hp concentrations before inoculation and until 8 h postinoculation were not observed with this detection system. Eckersall et al. (2001) showed elevated Hp milk concentrations in cows with naturally occurring mild and moderate mastitis. In the case of mild mastitis, 3 of 16 cows and, in moderate mastitis 1 of 13 cows, did not show detectable milk Hp concentrations. The detection limit of the immuno-diffusion assay used was 0.02 mg/mL . Due to these limitations, the use of milk Hp measurements for the diagnosis of mastitis was impractical. The ELISA reported herein is highly sensitive and offers

Table 1. Haptoglobin concentration (mean $\pm$ SEM) in blood and milk before and after intramammary LPS application in 6 cows.

	0 h	3 h	6 h	9 h	12 h
Blood ($\mu\text{g/mL}$)	32.8 $\pm$ 20.0	23.3 $\pm$ 9.0	58.3 $\pm$ 29.6	228.3 $\pm$ 42.2*	371.7 $\pm$ 37.5*
Milk, treated quarter ($\mu\text{g/mL}$)	0.9 $\pm$ 0.1	31.8 $\pm$ 9.2*	76.6 $\pm$ 25.1*	126.4 $\pm$ 23.8*	152.2 $\pm$ 22.6*
Milk, control quarter ($\mu\text{g/mL}$)	1.8 $\pm$ 0.4	4.1 $\pm$ 1.9	2.2 $\pm$ 0.4	2.5 $\pm$ 0.8	2.4 $\pm$ 1.1

* $P < 0.05$ compared with 0 h.

the opportunity to establish Hp levels in milk from clinically healthy cows throughout lactation and physiologically increased concentrations, for example, in colostrum. Further investigations on milk Hp in different cases of mastitis including subclinical mastitis are essential for advancing Hp measurements as a diagnostic tool for mastitis.

The early increase of the Hp concentration in milk in our own studies lead us to hypothesize that Hp may be synthesized locally in the mammary gland and thus not limited to changes in the permeability of the blood milk barrier as suggested by Eckersall et al. (2001). The extrahepatic synthesis of Hp has not been reported in cattle so far, although there are reports of Hp mRNA expression in adipocytes in LPS-treated mice (Friedrichs et al., 1995). The evidence of Hp mRNA that we observed in mammary gland tissues (the teat, the cisternal region, and the glandular parenchyma) supports the notion that the bovine mammary gland can be considered as another extrahepatic source of Hp. In addition, the demonstration of the LPS-induced upregulation of Hp mRNA strongly indicates that mammary Hp expression is stimulated by proinflammatory stimuli as it is in the liver. The relatively small increase of Hp mRNA we observed in the control quarters is probably due to a locally induced acute phase response as a result of the biopsy procedure itself, as suggested

by Schmitz et al. (2004). The authors reported similar increases and relations between control quarters and treated quarters for the proinflammatory cytokine tumor necrosis factor alpha.

The cellular localization of Hp expression in the mammary gland is not known yet, but migrated leukocytes might contribute or solely account for the Hp mRNA detected. In humans, Hp is concentrated within granulocytes; however, Hp gene transcription in granulocytes is negative, as shown by northern blotting analysis (Wagner et al., 1996). Only few data are available on the expression of other APP in the mammary gland. Eckersall et al. (2001) suggested that there might be a local synthesis of serum amyloid A during mastitis. This is supported by reports on the expression of mammary-associated serum amyloid A3 (McDonald et al., 2001) and mammary serum amyloid A3 homologue (Molenaar et al., 2002). Molenaar et al. (2002) reported high levels of serum amyloid A3 homologue mRNA in ductal epithelial cells and low levels in lactating secretory cells by in situ hybridization.

The biological function of Hp synthesis in the mammary gland during mastitis might be related to the bacteriostatic properties of Hp (Eaton et al., 1982). Haptoglobin blocks the hemoglobin-driven growth of *E. coli*

Figure 3. Haptoglobin PCR products (174 bp) demonstrated by RT-PCR in RNA extracts (2% agarose gel stained by ethidium bromide), M = molecular weight marker ϕ 174 DNA / BsuRI (HaeIII); L = liver, CR = cisternal region, GP = glandular parenchyma, T = teat, C = control. **Figure 4.** Haptoglobin mRNA expression in mammary tissue biopsies in the control quarters (♦) and the treated quarters (■) after LPS application (mean $\pm$ SEM, $n = 6$) in relation to ubiquitin expression.

in mice in vivo by preventing the use of hemoglobin iron. It thus may support or complement the actions of lactoferrin. Nevertheless, it is yet unknown whether Hp is able to prevent iron use in pathogens causing mastitis other than *E. coli*. *Staphylococcus aureus*, an important pathogen in bovine mastitis, exhibits multiple iron uptake systems, including hemin (Diarra et al., 2002). Moreover, *Neisseria meningitidis*, a pathogen in humans, is able to acquire iron from hemoglobin-Hp complexes in vitro (Lewis and Dyer, 1995).

In conclusion, our studies demonstrate that Hp is synthesized within the mammary gland. The LPS-induced increase of Hp mRNA supports a very close link between mastitis and Hp synthesis in mammary tissue and indicates that Hp detectable in milk not only originates from circulation. Using sufficiently sensitive assays as the ELISA we developed, milk Hp measurements may provide an additional tool for the investigation of inflammatory processes in the mammary gland and may also be used as an early diagnostic marker for mastitis.

ACKNOWLEDGMENTS

Parts of this project were supported through the Bundesministerium für Wirtschaft und Technologie, Germany, and through Biofocus GmbH, Recklinghausen, Germany. We would like to thank Isabella Israel for her excellent technical assistance.

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