Transcriptional profiling of the bovine hepatic response to experimentally induced \textit{E. coli} mastitis

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\textbf{Jørgensen HBH, Buitenhuis B, Røntved CM, Jiang L, Ingvartsen KL, Sørensen P.} Transcriptional profiling of the bovine hepatic response to experimentally induced \textit{e. coli} mastitis. \textit{Physiol Genomics} 44: 595–606, 2012. First published April 10, 2012; doi:10.1152/physiolgenomics.00084.2011.—The mammalian liver has several vital functions in metabolism, hormone synthesis, detoxification, and regulation of blood glucose levels to ensure homeostasis in the entire body. The elimination of endogenous and exogenous compounds that may otherwise perturb homeostasis is generally considered in two steps: phase I and phase II metabolism. Phase I metabolism is catalyzed mainly by the P450 superfamily of enzymes (35, 39), while phase II metabolism is catalyzed by e.g., UDP-glucuronosyltransferases (UGT) (38, 39). In addition, the liver plays a crucial role in the acute phase response (APR) to intruding pathogens (44). The hepatic APR is often initiated as a systemic reaction to cytokines produced at distant sites of infection (11), and several factors such as cytokines, leptin, and insulin influence the regulation of both metabolic and immune-related processes (31).

Disease by gram negative bacteria has been mimicked by the use of their surface lipopolysaccharide (LPS) in numerous studies. In beef cattle, \textit{Escherichia coli} infection has been mimicked by \textit{E. coli}-derived LPS to study e.g., respiratory diseases (10) and nitrogen uptake during immunological stress (47). In dairy cattle, LPS has been used to mimic \textit{E. coli}-induced mastitis, and it has been found to induce a systemic APR with increased levels of acute phase proteins in blood serum (25). At the transcriptome level, expression of genes coding for several inflammatory cytokines and acute phase proteins increased in the liver following intramammary LPS stimulation (46, 26). This change in transcription occurred simultaneously with well-known reactions to bacterial infection in cattle, such as fever and leukocytosis (10). Here we examined whether mastitis induced by \textit{E. coli} infection in the mammary gland of dairy cattle gives rise to a similar hepatic systemic response at the gene transcription level. After verifying the systemic response by observing clinical and paraclinical traits, we studied gene transcription in liver biopsies. The biopsies might contain both hepatocytes and Kupffer cells (macrophages that reside in the liver), and we hypothesized that the gene transcription profiles would reflect effects of \textit{E. coli} mastitis on biochemical processes in both cell types. In addition, we compared the transcriptional responses, hypothesizing that LPS is an adequate \textit{E. coli} mimic in experiments that aim to study hepatic systemic responses to infection.

\textbf{MATERIALS AND METHODS}

\textbf{Animals and \textit{E. coli} inoculation.} Sixteen healthy, primiparous Danish Holstein-Friesian cows were chosen for this study based on their general health and their udder health. Body temperature was normal; white blood cell count was within the normal range and tested negative in a glutaraldehyde test. The somatic cell count (SCC) in fore milk samples biosampled for the study reported in Buitenhuis et al. (8) was <27,000 cells/ml. Results from a Californian mastitis test were \textless 2 (Kruuse, Marslev, Denmark) (range 1–5), and they were free from major mastitis-causing pathogens. The cows were inoculated with \textit{E. coli} in one front quarter of the udder 4–6 wk after parturition. We used the Danish field isolate, \textit{K2b2}, of \textit{E. coli}. This isolate was derived from a cow with severe, acute mastitis. Prior to inoculation, \textit{E. coli} was grown in heart infusion medium [brain heart infusion (BHI); Merck, VWR - Bie & Berntsen] at 37°C overnight. The following day, the bacteria suspension was tested for purity and transferred to new BHI medium in a 10% dilution. The suspension was then centrifuged at 14,000 relative centrifugal force, and the resulting bacterial pellet was resuspended in endotoxin-free 0.9% NaCl. A dilution series was made from this resuspension, and the number of \textit{E. coli} bacteria present in each dilution was counted using the plate count agar method. After overnight incubation, the numbers of colony-forming bacteria were counted. The suspension was then diluted to contain approximately 20–40 colony-forming units (CFU) per ml. Prior to inoculation, \textit{E. coli} went through a period of perturbations to its normal homeostatic condition during the first 24 h following the intramammary stimulation to mimic infections by gram negative bacteria.

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RNA extraction. Biopsies collected after time liquid nitrogen, brought to the laboratory, and stored at biopsy. The liver tissue samples were immediately flash-frozen in was 14 gauge with a 17 mm notch and held 10 – 15 mg tissue per needle of the biopsy pistol ped for muscle biopsies in humans (Manan Automatic Biopsy System; 12 according to the procedure described in Andersen et al. (1) and in matic surface of the right hepatic lobe between ribs numbers 11 and minimally invasive liver biopsies was sampled below the diaphrag- post inoculation; and at 12 h intervals up to 300 h was recorded at 36, 12, and 6 h preinoculation; at time of inoculation; measured at 6 h preinoculation, at time of inoculation, and at 3, 6, 12, transcripion measured preinfection. for the three postinfection time points were compared with the postinfection - after acute immune response. Gene transcription levels 3 acute phase,

Clinical and paraclinical measures and sampling. A time series of minimally invasive liver biopsies was sampled below the diaphragmatic surface of the right hepatic lobe between ribs numbers 11 and 12 according to the procedure described in Andersen et al. (1) and in Vels et al. (46). Samples were collected using a biopsy pistol developed for muscle biopsies in humans (Manan Automatic Biopsy System; Marmon/MDTech, Gainesville, FL). The needle of the biopsy pistol was 14 gauge with a 17 mm notch and held 10 – 15 mg tissue per biopsy. The liver tissue samples were immediately flash-frozen in liquid nitrogen, brought to the laboratory, and stored at −80°C until RNA extraction. Biopsies collected after time = 0 were sampled below previous biopsies. The biopsies were collected at four time points: 1) 144 h preinfection - healthy, 2) 12 h postinfection - early acute phase, 3) 24 h postinfection - late acute phase, and 4) 192 h postinfection - after acute immune response. Gene transcription levels for the three postinfection time points were compared with the transcription measured preinfection.

SCC, representing the number of leukocytes in the milk, was measured at 6 h preinoculation, at time of inoculation, and at 3, 6, 12, 18, 24, 60, 84, 132, 180, and 228 h postinoculation. Body temperature was recorded at 36, 12, and 6 h preinoculation; at time of inoculation; at 3, 6, and 12 h postinoculation; and at 12 h intervals up to 300 h postinoculation. CFU, representing the number of Escherichia coli bacteria in milk samples, was estimated from 6 h preinoculation, at time of inoculation, and time points identical to body temperature recordings with an additional time point at 396 h postinoculation according to the procedure presented in Buitenhuys et al. (8).

Transcription profiling by microarrays. RNA extraction and labeling, as well as microarray hybridization and scanning procedures, were performed as described in Kristensen et al. (27). Briefly, total RNA was extracted from liver biopsies using the RNA-zol B RNA isolation methods (WAK-Chemie Medical). We used 5 μg of total RNA as template for first- and second-strand cDNA synthesis with the SuperScript Choice System (Life Technologies) according to the manufacturer’s instructions, except when using an oligo-dT primer containing a T7 RNA promoter site. Using the BioArray High Yield RNA Transcript Labeling Kit (Enzo, Farmingdale, NY), we then prepared biotin-labeled cDNA. We loaded 15 μg of the labeled cDNA onto the probe array cartridge of the Bovine Genome Array (Affymetrix, Santa Clara, CA). The Bovine Genome Array contains 24,128 probe sets representing 15,264 UniGene transcripts (annotation from May, 2006) for genome-wide analyses of transcription levels, and an annotation for the array is available from the NetAffx Analysis Center (Bovine.na29.annot.csv). In addition, we obtained updated annotation information from the Ensemble database, using the biomart package (version 2.0.0) in R (12). In total, 10,401 probes on the array had an Ensemble ID, and 9,369 probes could be assigned to biological processes in the Gene Ontology (GO) database. One microarray was analyzed for each cow at each sampling time, except that two arrays from 192 h had to be discarded (n = 54).

Statistical analyses. The data were analyzed with R (version 2.10.0) (http://www.r-project.org/). Gene transcription values for the liver samples were normalized using the robust multiarray average algorithm (24) as implemented in the Affy package (version 1.22.1). Descriptions of the microarray experiment and data are available from National Center for Biotechnology’s Gene Expression Omnibus data repository (13, 5) through accession number GSE25319. (reviewer’s link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25319).

Fig. 1. Mean recordings for clinical and paraclinical symptoms. Recordings of 3 clinical and paraclinical traits for 16 Danish Holstein-Friesian cows prior to inoculation and postinoculation with Escherichia coli in the udder. The 3 traits were body temperature (A), somatic cell count (the number of leukocytes in milk samples) (B), and colony-forming units (CFU; number of E. coli bacteria in milk samples) (C). Red triangles indicate time points at which liver biopsies were sampled. Blue circles illustrate standard deviations from the mean. Standard deviations for the 2 highest CFU counts in C are ± 117,222 and ± 118,650 (not shown in the graph).
Using a linear modeling approach (limma, version 2.18.2), we identified transcripts whose transcription levels had significantly increased or decreased at one or more time points following infection. Transcripts were defined as significantly differentially expressed (DE) when the Bonferroni-adjusted \( p \leq 0.05 \) for the overall time series and when fold-change in transcription compared with preinoculation was either \( >2 \) or \( < -2 \) at one or more time points. Using these two criteria, we picked out genes that both were significantly differentially transcribed based on a conservative test and had a large change in transcription level, maximizing our confidence in the resulting gene list.

We ran a 2-D cluster analysis based on the transcripts defined as significantly differentially transcribed using the heat map 2 option (gplots, version 2.18.2). This hierarchical clustering was based on Pearson’s correlation coefficients as distance measures. The number of clusters was based on two criteria: 1) it should result in a distinctly lower within-between cluster ratio for transcription variance than one fewer cluster and 2) no cluster should contain \(< 10\) genes. We tested for overrepresentation of GO categories in the defined clusters. Each cluster (edited to contain only one copy of each transcript and only annotated genes) was tested against a background of all annotated genes on the microarray using functional annotation clustering in the DAVID bioinformatics database (23).

Significantly increased or decreased transcription levels at specific time points following infection, using a linear modeling approach as above, were found by testing transcription levels at each of the three postinfection time points separately against preinfection transcription levels. The transcripts were defined as significantly expressed when the Bonferroni-adjusted \( p \leq 0.05 \) and fold-change in transcription was either \( >2 \) or \( < -2 \) compared with preinfection. This approach was included to enable comparisons between experiments as described below.

**Comparison and verification of results.** The present study was a follow-up to a study reported in Vels et al. (46) and Jiang et al. (26) where mastitis was induced by \( E. coli \)-derived LPS stimulation. The expectation was that the responses to LPS and \( E. coli \) would be very similar, perhaps with differences in timing. This way we were able to make a general comparison of the APR in bovine liver at the gene transcription levels from the microarray results in the two experiments.

Systemic responses to inoculation in the udder were compared between studies using LPS and \( E. coli \) to induce the response. This way we evaluated the suitability of LPS as an \( E. coli \) mimic in infection experiments. Since the samples from both studies were run on the Bovine Genome Array (Affymetrix), we were able to make direct comparisons. The DE transcripts identified in this study were tested against transcripts from liver samples collected at 22 h prior to inoculation and 3, 6, 9, 12, and 48 h following LPS stimulation (26). Significantly DE transcripts at specific time points in the LPS study were defined using the same criteria as for specific time points in this study. Pooling the results from both studies, a total of 1,520 transcripts were DE at any of eight samplings: three sampling times following \( E. coli \) inoculation and five sampling times following LPS stimulation. The \( t \)-statistics for the 1,520 DE transcripts from all of the eight samplings in the two studies were used for 2-D hierarchical clustering analysis as above, using the centered Pearson’s method.

Since the responses to \( E. coli \)-derived LPS and to \( E. coli \) were expected to be similar, we chose to verify the results from the present study by comparing transcription levels following stimulation/inoculation for key genes: activating transcription factor (ATF)3, Fos, JunB.
RESULTS

Verifying the systemic response to E. coli. E. coli was verified to be the causal inflammatory agent by recording three clinical and paraclinical symptoms. The presence of E. coli in the udder was verified by the count of CFU, peaking at 24 h following inoculation (Fig. 1C). Increased numbers of leukocytes in milk samples showed that the cows initiated an immune response to the bacteria in the udder with a peak SCC at 18–24 h following inoculation (Fig. 1B). In addition, average body temperature rose following E. coli inoculation and peaked after 14–18 h (Fig. 1A). These results allowed us to assume that any systemic responses in the liver samples were due to E. coli-induced mastitis.

Identifying distinct transcription profiles. Contrasting transcription levels postinfection with transcription levels preinfection, we found that 408 transcripts changed significantly over the time series. These transcripts were assigned to three clusters based on the correlation between their transcription profiles. Cluster one contained 107 DE transcripts (representing 69 unique gene IDs), cluster two contained 97 DE transcripts (representing 75 unique gene IDs), and cluster three contained 204 DE transcripts (representing 120 unique gene IDs) (Supplemental Table S1). The within-between ratio of the variance among transcription profiles was 0.27. We decided not to assign the transcripts to a larger number of clusters since this would invariably result in a cluster size of one. A heat map shows the transcription levels for the transcripts assigned to the three clusters (Fig. 2). The liver samples were grouped on the x-axis of the heat map according to similarity of transcription patterns. In general, samples from 12 h and 24 h postinfection had adjoining locations on the x-axis, while samples from 144 h preinfection and 192 h postinfection were interspersed among each other.

Transcription profiles for the three clusters of transcripts are presented in Fig. 3 and can be easily distinguished: Transcripts in cluster one showed increased transcription following E. coli inoculation and their transcription peaked at 12 h postinfection. In cluster two, transcription levels also increased following infection but peaked at 24 h postinfection. Transcription levels in cluster three decreased following infection with a minimum transcription level at 24 h postinfection. The transcription levels in all clusters were close to their initial levels at 192 h postinfection, indicating that the response to E. coli was at an end.

In general, genes coding for factors involved in transcription regulation were significantly overrepresented in cluster one (Table 1; Supplemental Table S2, column “Genes”; Supplemental Table S11). Key genes included in this cluster are ATF3, Fos, JunB, Jun, CEBPD, CREM, Bcl-2-associated athanogene 3 (BAG-3), PIM-1, GADD153, Myc, HSPH1, HSPA1A/HSPA1B. These results show that the E. coli infection in the udder led to a systemic response within 12 h.
postinfection. In cluster two, there was a significant overrepresentation of genes involved in responses to oxidative stress (Table 1; Supplemental Table S3, column “Genes”; Supplemental Table S11). Key genes included in cluster two are S100A8, S100A9, S100A12, GPX1, GPX3. The transcription of genes in cluster two peaked at 24 h postinfection and thereby coincided with the decrease in transcription seen in cluster three. Cluster three contained mainly genes coding for factors involved in breakdown processes, digesting e.g., sugars, amino acids, and hormones (Table 1, Supplemental Table S4). Cluster three included the key genes CYP2C19, CYP2E1, UGT1A1, UGT2A3. Thus, metabolic processes in the liver were suppressed following intramammary E. coli infection. Due to the variation in clinical traits among the individual animals and since we have few sampling times, we ran a functional annotation analysis on the entire list of DE genes to see the results since we have few sampling times, we ran a functional annotation clustering in DAVID (23). The entire bovine microarray was used as background, stringency for associating genes with a functional group was set to “high”, and the cut-off enrichment score was set to 1.3. Average transcription profiles for the 3 clusters are shown in Fig. 3.

Biological processes represented by gene ontology categories that were defined as significantly overrepresented in the 3 clusters of differentially expressed (DE) genes. The results are based on functional annotation clustering as implemented in DAVID (23). The entire bovine microarray was used as background, stringency for associating genes with a functional group was set to “high”, and the cut-off enrichment score was set to 1.3. Average transcription profiles for the 3 clusters are shown in Fig. 3.

**Table 1. Gene enrichment in clusters of DE genes**

<table>
<thead>
<tr>
<th>Name of Functional Group</th>
<th>Group Enrichment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Leucine zipper DNA binding</td>
<td>8.25</td>
</tr>
<tr>
<td>2. Regulation of cell death</td>
<td>3.81</td>
</tr>
<tr>
<td>3. Transcription regulation</td>
<td>3.62</td>
</tr>
<tr>
<td>4. Transcription regulation II</td>
<td>3.39</td>
</tr>
<tr>
<td>5. Negative regulation of cell death</td>
<td>2.97</td>
</tr>
<tr>
<td>6. Nuclear and organelle lumen</td>
<td>2.53</td>
</tr>
<tr>
<td>7. Stress response and heat shock protein 70</td>
<td>2.38</td>
</tr>
<tr>
<td>8. Positive regulation of biosynthetic processes and transcription</td>
<td>1.54</td>
</tr>
</tbody>
</table>

**Cluster 2**

<table>
<thead>
<tr>
<th>Name of Functional Group</th>
<th>Group Enrichment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Calcium binding</td>
<td>2.85</td>
</tr>
<tr>
<td>2. Protein complex assembly</td>
<td>2.05</td>
</tr>
<tr>
<td>3. Response to reactive oxygen and oxidative stress</td>
<td>1.62</td>
</tr>
</tbody>
</table>

**Cluster 3**

<table>
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<tr>
<th>Name of Functional Group</th>
<th>Group Enrichment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glucuronosyltransferase</td>
<td>3.46</td>
</tr>
<tr>
<td>2. Sugar and hormone metabolism</td>
<td>3.38</td>
</tr>
<tr>
<td>3. Amino acid breakdown</td>
<td>2.30</td>
</tr>
<tr>
<td>4. Cytochrome P450 and heme</td>
<td>2.23</td>
</tr>
<tr>
<td>5. Peroxisomes</td>
<td>2.07</td>
</tr>
<tr>
<td>6. Cholesterol and sterol biosynthesis</td>
<td>1.90</td>
</tr>
<tr>
<td>7. Glycerol and alditol metabolism</td>
<td>1.73</td>
</tr>
<tr>
<td>8. Skin development</td>
<td>1.56</td>
</tr>
<tr>
<td>9. Vitamin binding and transferase activity</td>
<td>1.42</td>
</tr>
</tbody>
</table>

**Table 2. Enrichment of DE genes from specific time points**

<table>
<thead>
<tr>
<th>Name of Functional Group</th>
<th>Group Enrichment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Leucine zipper DNA binding</td>
<td>8.92</td>
</tr>
<tr>
<td>2. Transcription regulation</td>
<td>3.61</td>
</tr>
<tr>
<td>3. Transcription</td>
<td>3.32</td>
</tr>
<tr>
<td>4. Regulation of cell death</td>
<td>3.11</td>
</tr>
<tr>
<td>5. Nuclear and organelle lumen</td>
<td>3.08</td>
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<tr>
<td>6. Stress response and heat shock protein 70</td>
<td>2.70</td>
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<tr>
<td>7. Negative regulation of cell death</td>
<td>2.52</td>
</tr>
<tr>
<td>8. Calcium binding</td>
<td>2.09</td>
</tr>
<tr>
<td>9. Positive regulation of biosynthesis and transcription</td>
<td>1.30</td>
</tr>
</tbody>
</table>

**Early and late APR.** When transcription levels preinoculation and postinoculation were contrasted, the largest number of transcripts with differential transcription was observed 24 h postinfection (n = 306), during the late APR (Supplemental Table S6). At 12 h postinfection, during the early APR, 144 transcripts were DE compared with preinfection (Supplemental Table S6), and 42 of them were overlapping with the list of DE transcripts from 24 h postinfection (Fig. 4). At 192 h postinfection, only five transcripts had different transcription levels compared with preinfection (Supplemental Table S6). None of these transcripts were identical to the DE transcripts from the other postinfection time points (Fig. 4). These results

**Fig. 4. Overlaps between lists of differentially expressed transcripts at specific time points.** Of the total of 408 differentially expressed (DE) transcripts, the majority of the transcription activity was found at 12 and 24 h postinfection, and there was an overlap in DE transcripts between the 2 time points. There were few DE transcripts at time 192 postinfection, and they were different from the DE transcripts at earlier time points. This indicates that the systemic response to E. coli infection in the udder was already active at 12 h postinoculation, that the response was progressive between 12 and 24 h, and that the response had come to an end at 192 h postinoculation.
correspond well with the heat map (Fig. 2) in that the tran-
scription levels of transcripts 192 h postinfection were similar
to their preinfection levels.

Time point-specific tests supported the above results (Table 2):
At 12 h postinfection, there was an overrepresentation of DE
genes assigned to the same functional groups as in cluster one
and two (Supplemental Table S7). The transcription profile for
cluster one was characterized by a peak transcription level at
12 h postinfection. At 24 h postinfection, there was an over-
representation of genes assigned to the same functional groups
as cluster two and three (Supplemental Table S8). Transcription
profiles for these two clusters both take extreme values at 24 h

Fig. 5. Clustering of samples from the present study and samples collected after LPS stimulation. This heat map was based on a pool of all 1,520 transcripts that were defined as significantly differentially expressed in the 8 samples [3 from the present study and 5 from Jiang et al. (26)], where the cows were inoculated with \textit{E. coli}-derived LPS in the udder. A hierarchical clustering of the samples showed a group to the left comprising 3 late acute phase samples, in the center 3 early acute phase samples, and to the right 2 samples collected after the acute phase had passed.
postinfection. Only five transcripts were differentially transcribed at 192 h postinfection (Supplemental Table S6).

Assessing the use of LPS as an E. coli mimic. Clustering the five samples from Jiang et al. (26) and the three samples from the present study based on the pool of DE transcripts shows a grouping of early acute phase samples, later acute phase samples, and samples taken after the immune response (Fig. 5). When the lists of DE transcripts from all samples (Table 3) are compared, the response to LPS at 6 h and the response to E. coli at 12 h postinoculation shared the greatest number of DE transcripts (overlapping transcripts made up 94% of the DE transcripts found for the E. coli response at this time point). The response to LPS at 12 h, in turn, showed the greatest similarity with the response to E. coli at 24 h postinoculation (overlapping transcripts made up 85% of the DE transcripts found for the E. coli response at this time point). Based on this, our gene transcription results indicate that the response to LPS is faster than to E. coli. Functional annotation clustering of the annotated genes from the two overlapping gene lists representing early and late acute phases showed that several of the significantly overrepresented functional groups from 12 and 24 h in this study were also overrepresented in the lists of genes that were in common with the LPS response (Supplemental Tables S7 and S8).

Transcription profiles for 17 key genes are graphed in Figs. 6–8 and show similar patterns in responses to LPS and E. coli. By graphical inspection, we see that the increase in transcription levels for ATF3, Fos, JunB, and the two S100 genes is higher in response to E. coli. However, the decrease in transcription of the CYP and UGT genes were more pronounced in response to E. coli.

**DISCUSSION**

The initial site of infection in mastitis is the mammary tissue. Still, the liver plays an important part in the systemic response to intruding bacteria: Proinflammatory cytokines IL-1β, IL-6, and TNF-α will stimulate hepatocyte production and release of complement factors and acute phase proteins, e.g., CRP, SAA, MBL, that will participate in the activation of innate immune responses, systemic inflammation, and tissue repair (36). In addition, cytokines from the liver induce the production and release of, e.g., neutrophils from the bone marrow to be directed toward tissue containing neutrophil attractants (36).

The inflammation caused by intramammary infection by E. coli induces an increased transcription in udder tissue of genes coding for chemokines that are known to attract neutrophils (8, 33). When neutrophils arrive in the mammary tissue, they will neutralize and ingest bacteria and LPS (9). They will then become apoptotic, and the majority of them are removed by macrophages in situ (7) or excreted in the milk. Bovine Kupffer cells (liver-resident macrophages) will produce proinflammatory cytokines upon activation by bacterial LPS (50). It has, however, been argued that LPS will not enter circulation and that the systemic effects from inflamed mammary tissue are due to other mediators, such as TNF-α that was found in increased concentrations in blood samples following intramammary LPS infection/E. coli infection (22). The relatively low transcription levels for TNF-α and IL-6 in our biopsies suggest that an absence of LPS leads to no activation of Kupffer cells in the biopsies. The increased transcription levels of IL-1β and IL-10 during the early acute phase suggests that Kupffer cell transcription of these two cytokines was induced by other mediators than LPS or it may have been induced by low levels of LPS from the gut (44, 51). Alternatively, the biopsies may have contained a very small proportion of Kupffer cells and IL-β and IL-10 may be produced by other types of liver cells.

Transcription levels for four acute phase proteins changed following E. coli infection, indicating that the hepatocytes were activated by proinflammatory cytokines from a source different from hepatic Kupffer cells, e.g., mammary macrophages (28, 45). The transcription of two acute phase proteins, Hp and SAA3, increased four- to fivefold during the APR in this study (Fig. 8). This was expected, since these two proteins are used as indicators of mastitis in dairy cattle (34). The transcription of CRP increased significantly during the late acute phase of the response though not as much as Hp and SAA3 (Fig. 7). This suggests that it may be a moderate acute phase protein in cattle, but there is disagreement on this in the literature (34). Transcription of MBL2 actually decreased during the response, particularly during the late acute phase (Fig. 7), suggesting that MBL2 is not an acute phase protein in the hepatic systemic response to bovine mastitis.

Our genome-wide transcription analysis identified additional genes in the bovine liver that changed significantly following intramammary E. coli infection, and a number of key genes are discussed below.

**Regulation of transcription and cell death.** The functional group “leucine zipper/DNA binding” is the group of genes most overrepresented among the DE transcripts in the liver biopsies (Tables 1 and 2). The genes included in this group showed increased transcription with a peak at 12 h postinoculation, and they were also represented in other functional groups associated with regulation of transcription. A leucine zipper is a region found in a group of proteins that participate in signal transduction or form dimers and participate in regulation of gene transcription (21). This group of proteins includes mainly the JUN, the Fos, and the ATF families of proteins, and they may act as subunits of the AP-1 (activation protein 1) transcription factor (37, 21).

Genes encoding several AP-1 subunits were differentially transcribed in the liver samples, including: Fos, AFT3, JUN,

| Table 3. Overlaps between DE transcripts following inoculation by E. coli and LPS |
|----------------------------------|----------|----------|----------|----------|----------|
|                                 | E. coli 12 | E. coli 24 | E. coli 192 | LPS 3 | LPS 6 | LPS 9 | LPS 12 | LPS 48 |
|                                 | 144       | 42       | 306       | 0      | 0     | 0     | 5      | 0     |
| **E. coli**                     | 12        | 24       | 192       | 3      | 6     | 9     | 12     | 48    |
| **LPS**                         | 136       | 105      | 0         | 6      | 0     | 0     | 115    | 0     |
| **LPS**                         | 70        | 18       | 0         | 6      | 105   | 0     | 86     | 0     |
| **LPS**                         | 96        | 213      | 0         | 53     | 486   | 0     | 971    | 0     |
| **LPS**                         | 74        | 260      | 0         | 44     | 282   | 0     | 565    | 0     |
| **LPS**                         | 6         | 10       | 0         | 7      | 9     | 11    | 11     | 14    |

Values on the diagonal (shown in boldface) show the number of DE transcripts in each sample. Cells below the diagonal show the number of overlapping transcripts between pairs of different time points following inoculation with E. coli and LPS, respectively. Transcript lists in italics mark time points of maximum overlap, indicating similar phases in the systemic response to intramammary LPS and E. coli inoculation.
JunB (as well as CEBPD and CREM). Average transcription for each of the AP-1 subunits increased with a peak level during the early acute phase. The exact composition of the AP-1 transcription factor and its effect on gene transcription depend on a number of factors, including the type and strength of extracellular stimuli, cell type, and differentiation state. For example, JUN is affected by the proinflammatory cytokines TNF-α, IL-1β, and IL-6 and by oxidative stress (43, 49). In mouse liver, JUN plays an important role in the proliferation of liver cells (43). When JUN is not present, JunB can function as a positive growth regulator, but JUN-JunB heterodimers show antiproliferative activity (21). Transcription of ATF3 transcription is increased following hepatic injury, leading to hepatocyte proliferation (15). On the contrary, Fos was found to act as a tumor suppressor in human epithelial hepatocytes by inducing cell cycle inhibition and cell death (32). The fact that transcription levels for these genes returned to preinfection levels at 192 h indicates that the production of these AP-1 subunits for transcription regulation in the hepatocytes successfully ensured a return to liver homeostasis.

Other enriched functional groups clustered together with the AP-1 subunits, and two of them are associated with regulation of cell death. The groups include PIM-1 (pim-1 oncogene, a kinase), which is involved in the activation of regulatory proteins and the inactivation of proapoptotic proteins (4). GADD153 (DNA-damage-inducible transcript 3) interacts with other regulation factors to induce apoptosis in human liver cells (52). Myc [v-myc myelocytomatosis viral oncogene homolog (avian)] is involved in a large array of cellular processes and acts as a regulator for factors that promote both cell differentiation and cell death (18). These three genes were all transcribed at increased levels, particularly during the early acute phase of the systemic response to E. coli.

Also the chaperone proteins BAG3 and HSP70 showed an increased transcription during the early acute phase. The BAG3 protein promotes autophagy, the degradation of cellular components via lysosomes (14), and may be a weak apoptosis-inhibitor (2). As hubs linking proteins in networks, the HSP70 and other chaperones are thought to support system stability by acting as buffers. As cochaperones for the BAG protein family, represented here by BAG3, and able to modulate the extent of protein degradation (14), HSP70 may contribute to the down-regulation of apoptosis. Overall, a successful response to E. coli seems to include changes in transcription of cell death-

Fig. 6. Transcription profiles for ATF3, Fos, JunB, S100A8, and S100A9. Fold changes in transcription levels following LPS stimulation (dark bars) or E. coli inoculation (light bars) for key genes that associated with gene transcription regulation (ATF3, Fos, JunB) and oxidative stress response (S100A8, S100A9). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. All P values are time specific and unadjusted.
associated and chaperone genes that maintain a population of healthy hepatocytes by letting abnormal cells die while producing and maturing new cells.

Oxidative stress. Transcription of S100A8/S100A9 and S100A12 reached the highest levels of all the key genes studied here. They all reached peak transcription levels of 8- to 10-fold increases at 24 h following inoculation (Supplemental Table S11). As is the case here, S100A8 and S100A9 are often coexpressed (16, 29), suggesting common regulation of their transcription by e.g., the AP-1 (16), whose subunits also show increased transcription levels in this study. The S100 proteins A8, A9, and A12 are induced by different mechanisms in different cells and may act both pro- and anti-inflammatory (17). It has been suggested that the transcription of the oxidation-sensitive S100A8 and S100A9 proteins in activated macrophages that produce reactive oxygen species and the induction of their transcription by IL-10 (known to be an anti-inflammatory cytokine) indicates that S100A8 and S100A9 have anti-inflammatory properties, themselves (42, 17). Two genes coding for glutathione peroxidases (GPX1, GPX3) also showed peak transcription levels at 24 h postinoculation. These peroxidases metabolize hydrogen peroxide and a range of organic peroxides, thereby preventing oxidative damage to cells (3).

According to Goyette and Geczy (17), the S100 proteins are mainly expressed in cells at the site of inflammation. Our results show that S100A8, S100A9, and S100A12 (Fig. 6 and Supplemental Table S11) are also very actively transcribed in the bovine liver during systemic responses to infection in the udder. We propose that the increased transcription of these genes from the functional group called “response to oxidative stress” peaking at 24 h postinoculation suggests a clean-up of reactive oxygen species and metabolites in hepatocytes that may accumulate due to perturbations to normal liver function during the response to infection.

Perturbations to normal liver function. Among the genes with a significantly lowered transcription level following infection, two large gene families were represented in the enriched functional annotation groups. The first gene family is the CYP family of enzymes that catalyze oxidative conversions...
of both endogenous and exogenous compounds into either inactive products or cytotoxic metabolites (35). The CYP genes are normally transcribed in the bovine liver (41), and their activities are often referred to as phase I metabolism because their products are used as substrates for enzymes in a following phase II metabolism (30). The second large gene family that showed lowered transcription levels in the present study are important in phase II metabolism: UGT. UGT enzymes initiate the elimination of possibly toxic lipophilic compounds through the biliary and renal systems to prevent cellular accumulation (19).

Studies on cultured human and rat hepatocytes found that the transcription of several CYP genes was lowered by IL-1, IL-6, and TNF-α (20). This fits the hypothesis that during infection, hepatocytes will de-prioritize processes involved in their normal homeostatic activities (48). In this study, we found that transcription levels for both phase I and phase II enzymes decreased four- to sixfold during the late acute phase. This co-occurred with a peak transcription of genes associated with the functional group “response to oxidative stress,” suggesting that, e.g., the S100 and GPX gene products may be involved in a clean-up of reactive oxygen species and metabolites that have accumulated in hepatocytes during their altered activity during the APR to the mammary E. coli-infection.

Fig. 8. Transcription profiles for SAA3, Hp, CYP2C19, CYP2E1, UGT1A1, and UGT2A3. Fold-changes in transcription levels following LPS stimulation (dark bars) or E. coli inoculation (light bars) for key genes that encode acute phase proteins (SAA3, Hp) and genes associated with hepatocyte metabolism (CYP2C19, CYP2E1, UGT1A1, UGT2A3). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. All P values are time specific and unadjusted.
Assuming that similar transcription patterns will result from similar phases in the immune responses to LPS and *E. coli*, we would expect large overlaps in lists of DE transcripts and similar patterns of increased and decreased transcription levels for genes in cows that had reached similar phases of immune responses. Early acute phase samples and late acute phase samples from the two studies clustered together, but the test for similarities between DE transcripts at various time points was in line with the results from clinical and paraclinical trait measurements: the systemic response to intramammary inoculation with LPS was several hours faster than the response to *E. coli*. The systemic responses to *E. coli* and LPS also showed great similarities: The early acute phase at 12 h and 6 h postinoculation with *E. coli* and LPS, respectively, was characterized by an increase in transcription of genes that are, themselves, involved in transcription and regulation of transcription (Supplemental Table S9). The late acute phase at 24 h for *E. coli* and 12 h for LPS was characterized by genes involved in various metabolic processes and response to oxidative stress (Supplemental Table S10).

In general, the number of DE transcripts following LPS stimulation was higher, suggesting a stronger response. It may, however, be of greater importance that the functional groups of genes related to peroxisomes was third-ranking on the list of enriched functional groups at 24 h in this study, but it was absent among the overlapping genes (Table 2, Supplemental Tables S8 and S10), indicating a more limited regulation of peroxisome-related genes following LPS stimulation. Most of the peroxisome-related genes showed decreased transcription during the late acute phase (result not shown). This would be an advantage to the cow since it inhibits the production of reactive oxygen that could, in turn, cause oxidative stress in hepatocytes (40). In addition, transcription levels for the key metabolism-associated genes, CYP2C19, CYP2E1, UGT1A1, and UGT2A3 (Fig. 8, Supplemental Table S11) tend to show a more marked decrease in transcription levels in this study than following LPS stimulation. Hoeben et al. (22) concluded that the amounts of cytokines (specifically TNF-α) absorbed into circulation during *E. coli* infection was higher than during an LPS response. Unfortunately, it is not possible for us to compare blood concentrations of TNF-α in the two experiments, and it remains to be studied whether TNF-α (or other cytokines) has a major impact on the downward change in transcription levels for metabolism-associated genes in bovine hepatocytes during the APR to mastitis.

In conclusion, this study shows that the intramammary *E. coli* inoculation did induce a systemic APR in the cattle liver as was previously shown with LPS stimulation. This response includes an early increase in transcription levels for genes associated with gene transcription and regulation of cell death, followed by a later increase in transcription of genes associated with responses to oxidative stress, coinciding with a decreased transcription of genes coding for factors involved in metabolic processes. Comparing hepatic systemic responses to *E. coli* and LPS, we find that though they show large similarities, the timing of the acute phase is off, in agreement with Blum et al. (6) and Hoeben et al. (22). It seems that *E. coli* needs time to establish itself in the udder and produce a sufficient amount of LPS to induce a systemic immune response and that LPS stimulation induces fast response that also levels off fast due to clearance (6). Using LPS to mimic *E. coli* in infection trials examining hepatic processes should therefore be done with some caution: the general trends in the response may be quite similar, but the specific kinetics and exact biochemical processes of the response may not accurately mimic an *E. coli* response.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


