

5-Lipoxygenase, cyclooxygenase-2 and tumor necrosis factor alpha gene expression in somatic milk cells

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The goal of this work was to investigate whether different fractions of somatic cells in cow milk do produce prostaglandins, leukotrienes and the cytokine tumor necrosis factor alpha (TNF α) mRNA expressions of TNF α and of key enzymes of prostaglandin and leukotriene biosynthesis, cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO), respectively, were quantitatively determined. Eleven clinically healthy Brown Swiss cows were either defined as control (C) group (n=5; all quarters < 150 000 cells/ml) or as group with partially elevated quarter somatic cell counts (SCC; n=6) with at least 1 quarter > 150 000 cells/ml (H) and 1 quarter < 150 000 cells/ml (L). Total quarter milk from 1 quarter of control animals and from 2 quarters of cows with partially elevated SCC (1 of H and 1 of L) was collected. Cells were fractionized into macrophages and lymphocytes (MAC+LYM) and polymorphonuclear leukocytes (PMN) using a density gradient. RNA was isolated, reversely transcribed and quantitative PCR was carried out. mRNA expression was similar for 5-LO, COX-2 and TNF α : no differences between C and L quarters were detected. However, expression was markedly elevated in H quarters. mRNA expression of all parameters tested was higher in MAC+LYM than in the PMN fraction. In conclusion, this investigation underlines that somatic milk cells are able to synthesize 5-LO, COX-2 and TNF α .

5-Lipoxygenase, Cyclooxygenase-2 und Tumornekrosefaktor alpha als Expression in somatischen Zellzahlen

In der vorliegenden Arbeit wurde untersucht, ob die verschiedenen Typen somatischer Milchezellen Prostaglandine, Leukotriene und den Tumornekrosefaktor alpha (TNF α) produzieren. Hierzu wurde die mRNA-Expression von TNF α sowie von Schlüsselenzymen der Prostaglandin- und Leukotrien-Biosynthese, Cyclooxygenase-2 (COX-2) bzw. 5-Lipoxygenase (5-LO), quantitativ nachgewiesen. Elf klinisch gesunde Braunviehkühe wurden entweder der Kontrollgruppe zugeordnet (n=5), wenn in allen Vierteln die somatische Zellzahl (SCC) < 150 000 Zellen/ml lag (C), oder der Gruppe mit teilweise erhöhter immunologischer Aktivität, sofern die SCC mindestens in einem Viertel > 150 000 Zellen/ml (H) und in einem Viertel < 150 000 Zellen/ml (L) war. Die Gemelke eines Viertels der Kontrolltiere und zweier Viertel der Tiere mit teilweise erhöhtem SCC (je ein H und ein L) wurden gesammelt. Die Milchezellen wurden über einen Dichtegradienten in Makrophagen und Lymphozyten (MAC+LYM) bzw. polymorphkernige Leukozyten (PMN) fraktioniert. Nach Isolation und reverser Transkription der RNA wurde eine quantitative PCR durchgeführt. Die Expression aller untersuchten Faktoren wiesen keinen Unterschied in den C und L Vierteln auf, waren allerdings deutlich erhöht in den H Vierteln. Grundsätzlich war die mRNA Expression in den MAC+LYM deutlich höher als in den PMN. Wichtige Entzündungsmediatoren wie 5-LO, COX-2 und TNF α werden demnach zumindest teilweise von somatischen Milchezellen gebildet.

06 Milk somatic cells (expression of interleukins)

06 Milchezellen (Expression von Interleukinen)

1. Introduction

Within the mammary gland of dairy cows a substantial amount of immune cells has to be transported from blood circulation into milk (7, 12) to provide cellular immune defence. Inflammatory mediators, such as cytokines, prostaglandins and leukotrienes, exhibit potent chemokinetic and chemotactic activity for leukocytes (4, 8, 14, 17) and enhance the bactericidal activity of phagocytes in dairy cows (18). Moreover, they induce vascular permeability and hyperalgesia during inflammatory disorders (10, 26).

The goal of this work was to investigate to which extent different fractions of somatic cells at different somatic cell count (SCC) levels are able to synthesize leukotrienes and prostaglandins. Therefore, we developed a quantitative and highly sensitive reverse transcription (RT) polymerase chain reaction (PCR) method to determine the mRNA expression of the key enzymes in leukotriene and prostanoid (prostaglandins, prostacyclin and thromboxanes) biosynthesis, *i.e.* 5-lipoxygenase (5-LO) and cyclooxygenase-2 (COX-2). The expression of tumor necrosis factor alpha (TNF α), a cytokine known to be crucial during early inflammatory stages in the mammary gland (21, 24), has been studied concomitantly.

2. Materials and methods

2.1 Animals and milk collection

Eleven lactating Brown Swiss dairy cows with no clinical signs of mammary disease were investigated. Five animals were in their 1st lactation, 2 in their 2nd, 3 in their 3rd and 1 in its 8th lactation. SCC of total quarter milk was measured with a fluoro-opto-electronic method using a Fossomatic 5000 cell counter (Foss Electric, Hillerød, Denmark). Cows were defined as control group (n=5), if all quarters had < 150 000 cells/ml (C = control) and as group with partially elevated quarter SCC (n=6), if at least 1 quarter had > 150 000 cells/ml (H = high) and at least 1 quarter < 150 000 cells/ml (L = low). Total quarter milk (1–4.5 l) from 1 quarter of control group cows and from 2 quarters of cows with partially elevated SCC (1 of H and 1 of L) was collected at one morning milking.

2.2 Cell isolation and cell ratio evaluation

The milk was centrifuged for 30 min at 220 g. The cell pellet was washed 3 times in phosphate buffered saline (PBS) pH 7.4 and centrifuged for 15 min at 4°C and 220 g (11). Cell fractions were separated using a density gradient (3, 22, 25). Therefore, the cell pellet was

resuspended in 15 ml PBS and carefully layered on 10 ml LSM (Lymphocyte Separation Medium, ICN, Aurora, OH, USA) with solution density 1.077–1.080 g/ml at 20°C. After centrifugation (50 min, 4°C, 220 g) macrophages and lymphocytes (MAC+LYM) were distributed within the interphase, polymorphonuclear leukocytes (PMN) were located in the pellet. Viability of the collected cells was 60–80%, assessed by the trypan blue exclusion method. Cell ratio evaluation was performed using a Neubauer's counting chamber (Brand, Wertheim, Germany).

2.3 RNA isolation and cDNA synthesis

Total RNA was isolated using TriPure (Roche, Basle, Switzerland) according to the manufacturers instructions. In order to quantify the extracted RNA, the optical density was determined at 3 different dilutions of the final RNA preparations at 260 nm. RNA integrity was verified by ethidium bromide stained gel electrophoresis and by optical density (OD)₂₆₀/OD₂₈₀nm absorption ratio >1.75. 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.

2.4 Oligonucleotide primers and identification of bovine 5-LO

The primers of COX-2 (5'-CTCTTCCTCCTGTGCC TGAT-3' sense oligonucleotide at position 410 coding sequence [CDS]; 5'-CTGAGTATCTTTGACTGTGGGAG-3' antisense oligonucleotide at position 768 CDS; PCR product length 359 bp) and TNF α (5'-TAACAAGCCGG TAGCCACG-3' sense oligonucleotide at position 255 CDS; 5'-GCAAGGGCTCTTGATGGCAGA-3' antisense oligonucleotide at position 531 CDS; PCR product length 277 bp) were designed using published bovine nucleic acid sequences (EMBL Ac. No. AF031698, AF011926).

The bovine 5-LO sequence was unpublished. For PCR several oligonucleotide primer pairs were designed, according to published human, mouse and rat 5-LO sequences (EMBL Ac. No. E02572, L42198, J03960), using a multiple sequence alignment (6). PCR products were extracted from a 2% agarose gel using QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced (Toplab, Martinsried, Germany). Primers for the bovine 5-LO were designed based on this newly obtained nucleotide sequence (5'-GCCCTTCTA CAACGACTTCGA-3' sense oligonucleotide at position 120 CDS; 5'-CAGGGTCCACTCCATCCA-3' antisense oligonucleotide at position 451 CDS; PCR product length 332 bp).

2.5 Quantification by real-time PCR

Quantitative analysis of PCR products was carried out in the LightCycler (Roche Diagnostics, Basel, Switzerland) using external plasmid DNA standard dilutions derived from cloned specific cDNA fragments of 5-LO, COX-2 or TNF α according to PFAFFL (15). 2 μ l reversely transcribed total RNA (12.5 ng/ μ l) was used as PCR template. Specific PCR cycle conditions are listed in Table 1. To verify the specificity of each LightCycler PCR quantification system and to generate cDNA standards

the LightCycler amplicons were cloned into pCR 4.0 vector (Invitrogen, Groningen, NL) and sequenced.

To confirm a constant house keeping gene expression level in the investigated total RNA extractions derived from the MAC+LYM and PMN cell fraction, an ubiquitin (UbC) RT-PCR was performed in the LightCycler. The primers (5'-ATGCAGATCTTTGTGAAGAC-3' sense oligonucleotide at position 1 CDS; 5'-CTTCTGG ATGTTGTAGTC-3' antisense oligonucleotide at position 189 CDS; PCR product length 189 bp) were designed using a published bovine nucleic acid sequence (EMBL Ac. No. Z18245). UbC specific mRNA was quantified in the same way as all other parameters. Specific LightCycler PCR conditions are summarized in Table 1.

Table 1: Product specific LightCycler-PCR conditions

Cycle segment and duration (s)	Temperature (°C)			
	5-LO	COX-2	TNF α	UbC
Denaturation	5	95	95	95
Annealing	10	64	64	64
Elongation	25	72	72	72
Acquisition	3	85	81	86

2.6 Statistical evaluations

Differences between C and L quarters were tested for significance ($p < 0.05$) using Wilcoxon's rank sum test of the NPAR1WAY procedure of SAS (19). Differences between L and H quarters and differences between MAC+LYM and PMN cell fractions (within animal) were tested for significance ($p < 0.05$) by Wilcoxon's signed rank test of the UNIVARIATE procedure of SAS.

3. Results

3.1 Bovine 5-LO sequence

A 1280 bp fragment of the bovine 5-LO CDS (EMBL Ac. No. AJ306424) was identified. Compared with published sequences of other species, there are 90.7% homology to human 5-LO (position bp 10–1289 CDS), 86.3% homology to mouse 5-LO (position bp 10–1289 CDS) and 83.8% homology to rat 5-LO (position bp 10–1286 CDS).

3.2 mRNA expressions of 5-LO, COX-2 and TNF α

Mean SCC were 27 ± 5 and $34 \pm 20 \times 10^3$ /ml in C and L quarters, respectively, and significantly higher in H quarters ($741 \pm 289 \times 10^3$ /ml).

The efficiency of cell separation was visible in the amounts of total RNA of all investigated 17 quarters: MAC+LYM 3.58 ± 0.60 ng per 10^3 cells and PMN 0.55 ± 0.12 ng per 10^3 cells.

As shown in Table 2, mRNA expressions were elevated in H quarters as compared with L quarters for 5-LO, COX-2 and TNF α . However, there were no significant differences between C and L quarters. In one animal of group C, values were slightly higher than in the others. Therefore, differences between C and L were completely abolished if this animal was omitted from evaluation. Values for group C ($n=4$) were 5.4 ± 0.9 molecules/cell and $1.8 \pm 0.6 \times 10^3$ molecules/ml milk for 5-LO, 24.3 ± 8.1 molecules/cell and $10.0 \pm 3.9 \times 10^3$ molecules/ml milk for COX-2, and 81.0 ± 23.8 molecules/cell and $34.9 \pm 13.6 \times 10^3$ molecules/ml milk for TNF α .

Table 2: mRNA expression in somatic milk cell fractions (mean \pm SEM)

Cell fraction	Quarter	MAC+LYM			PMN		
		C (n=5)	L (n=6)	H (n=6)	C (n=5)	L (n=6)	H (n=6)
5-LO	Molecules/cell	27 \pm 22	8.9 \pm 3.7	22 \pm 3*	0.4 \pm 0.3	0.4 \pm 0.1	1.0 \pm 0.2*
	10 ³ Molecules/ml milk	12 \pm 11	8.7 \pm 6.6	461 \pm 142*	1.1 \pm 0.6	2.5 \pm 2.1	31 \pm 7*
COX-2	Molecules/cell	37 \pm 14	11 \pm 5	58 \pm 16*	0.4 \pm 0.2	0.3 \pm 0.1	1.5 \pm 0.3*
	10 ³ Molecules/ml milk	16 \pm 8	8 \pm 4	1649 \pm 811*	2.3 \pm 1.5	1.0 \pm 0.8	47 \pm 9*
TNF α	Molecules/cell	143 \pm 65	48 \pm 29	200 \pm 90*	2.6 \pm 0.9	1.7 \pm 0.7	8.8 \pm 2.4*
	10 ³ Molecules/ml milk	66 \pm 33	27 \pm 15	3924 \pm 1918*	8.2 \pm 2.5	5.8 \pm 3.7	260 \pm 66*

*Means of quarters "L" and "H" are significantly different ($p < 0.05$)

Calculated per ml of milk, mRNA expression of proteins tested in C and H quarters was significantly higher in MAC+LYM than in PMN cell fractions. There was also an elevation in L quarters, however not significant due to 2 animals with very low SCC (7 and 14 $\times 10^3$ /ml). In all quarters, mRNA expression of 5-LO and COX-2 per cell was significantly higher in MAC+LYM than in PMN cells. Accordingly, mRNA expression of TNF α in C and L quarters were significantly higher in MAC+LYM than in PMN cells, but not in H quarters.

3.3 UbC mRNA expression

UbC mRNA expression levels were quantified and based on the average UbC mRNA expression measured in all samples ($n=34$). In the MAC+LYM cell fraction UbC expression was 119% in H quarters, 65% in L quarters and 63% in C quarters. In PMN cell fraction expressions were similarly distributed: 196% in H quarters, 79% in L quarters and 66% in C quarters.

4. Discussion

This study was designed to investigate the source and expression levels of 5-LO, COX-2 and TNF α mRNA in milk cells. MAC, LYM and PMN are the 3 major cell types in cow milk; their number depends on the individual, stage of lactation and udder health state. During inflammatory processes, predominantly the number of PMN increases (16). TNF α is one of the acute phase cytokines produced during early stage of acute *Escherichia coli* (*E. coli*) mastitis (2, 20, 21). It modulates many host reactions to foreign antigens, like phagocytosis or recruitment of leukocytes by their chemotactic activity and by elevated expression of adhesion molecules on circulating leukocytes and endothelial cells (18, 20). As expected, we found the highest mRNA expression of TNF α in the H quarters, *i.e.* the quarters with increased immunological activity. In general, TNF α is mainly produced by macrophages and monocytes (13, 23). Accordingly, our study revealed that mRNA expression of TNF α was significantly higher in MAC+LYM than in PMN cell fractions in C and L, but not in H quarters. The H quarter of one animal showed a marginally higher mRNA expression of TNF α per cell in PMN, than in MAC+LYM. This somewhat unexpected finding might be due to the fact that no differentiation between acute and chronic stage of inflammation was possible.

An additional feature of TNF α is to induce the production of arachidonic acid metabolites, like prostaglandins and leukotrienes (9). They are involved in some events during acute and chronic inflammatory disorders in dairy cows, like increasing vascular permeability,

chemotaxis and hyperalgesia (4, 8, 10, 14, 17, 26). Key enzymes in prostanoid and leukotriene biosynthesis are COX-2 and 5-LO, respectively. One of the therapeutic opportunities in clinical mastitis is to modulate the activity of these enzymes by nonsteroidal anti-inflammatory drugs (NSAIDs). While the bovine COX-2 coding sequence was already known, the bovine 5-LO sequence needed to be identified before designing appropriate primers. Significantly elevated mRNA expression was detected for COX-2 and 5-LO in H quarters as compared with L quarters; however, no differences between C and L quarters were found. In the MAC+LYM cell fraction, mRNA expression of COX-2 per ml milk was nearly 100-fold higher in H quarters than in L and C quarters. This characteristic increase was likely to be due to increased immunological activity or inflammatory processes: an increasing number of milk cells per ml milk with simultaneously reduced milk production and, as suggested by our data, an increasing transcriptional rate per cell.

In our quantitative PCR analysis UbC mRNA expression in the samples tested showed minor differences. It has previously been demonstrated that even typical housekeeping genes like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can be up-regulated under certain circumstances (1). The UbC mRNA expression differences found in our study, were not significant and visible only due to the high sensitivity of the method using the LightCycler which detects differences in mRNA expression more sensitive than conventional RT-PCR followed by densitometric gel analysis.

Our results indicate that 5-LO, COX-2 and TNF α are, at least to a certain extent, produced by somatic milk cells, *i.e.* represent milk borne factors. Expression of each compound studied was locally elevated in quarters with increased immunological activity, *i.e.* higher SCC, indicating that the somatic milk cells themselves are involved in the maintenance of immune response in milk. Therefore it can be assumed that extremely low SCC in the milk of healthy glands causes reduced or delayed immune response to invading microorganisms. A threshold of too low SCC to allow adequate immune response is not known. However, an annual bulk milk SCC <150000 cells/ml has been shown to be associated with a higher rate of clinical mastitis, than annual bulk milk SCC \geq 150000 cells/ml milk (5). We did not find any differences in mRNA expression between C and L quarters. This is a confirmation on the mRNA level, that increasing immunological activity within the mammary gland is a local effect restricted to single quarters. However, most likely factors important to induce immune response are also synthesized by mammary epithelial cells. Further investigations are in progress.

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5. References

- (1) BERETA, J., BERETA, M.: *Biochem. Biophys. Res. Commun.* **217** 363–369 (1995)
- (2) BLUM, J.W., DOSOGNE, H., HOEBEN, D., VANGROENWEGHE F., HAMMON, H.M., BRUCKMAIER R.M., BURVENICH, C.: *Domest. Anim. Endocrinol.* **19** 223–235 (2000)
- (3) CONCHA, C., HOLMBERG, O., MOREIN, B.: *J. Dairy Res.* **45** 287–290 (1978)
- (4) CRAVEN, N.: *Comp. Immun. Microbiol. Infect. Dis.* **9** 29–36 (1986)
- (5) ELBERS, A.R.W., MILTENBURG, J.D., DE LANGE, D., CRAUWELS, A.P.P., BARKEMA, H.W., SCHUKKEN, Y.H.: *J. Dairy Sci.* **81** 420–426 (1998)
- (6) FOX, A., MEEUSEN, E.: *Vet. Immunol. Immunopathol.* **68** 293–296 (1999)
- (7) HARMON, R.J., HEALD, C.W.: *Amer. J. Vet. Res.* **43** 992–998 (1982)
- (8) HEIDEL, J.R., TAYLOR, S.M., LAEGREID, W.W., SILFLOW, R.M., LIGGITT, H.D., LEID, R.W. *Am. J. Pathol.* **134** 671–676 (1989)
- (9) HOECK, W.G., RAMESHA, C.S., CHANG, D.J., FAN, N., HELLER, R.A.: *Proc. Natl. Acad. Sci. USA* **90** 4475–4479 (1993)
- (10) KOENIG, W., SCHOENFELD, W., RAULF, M., KOELLER, M., KNOELLER, J., SCHEFFER, J., BROM, J.: *Eicosanoids* **3** 1–22 (1990)
- (11) LIEBE, A.: *Diss. Techn. Univ. Munich-Weihenstephan, Germany* (1996)
- (12) NICKERSON, S.C., PANKEY, J.W.: *J. Dairy Sci.* **67** 826–834 (1984)
- (13) PAULI, U.: *Crit. Rev. Eukaryot. Gene Expr.* **4** 323–344 (1994)
- (14) PERSSON, K., LARSSON, I., HALLÉN SANDGREN, C.: *Vet. Immunol. Immunopathol.* **37** 99–112 (1993)
- (15) PFAFFL, M.W., HAGELEIT, M.: *Biotechnol. Lett.* **23** 275–282 (2001)
- (16) RAINARD, P., RIOLLET, C., POUTREL, B.: In *Proc. IDF Symp. on Immunology of Ruminant Mammary Gland*, Stresa, Italy, 212–218 (2000)
- (17) ROSE, D.M., GIRI, S.N., WOOD, S.J., CULLOR, J.S.: *Am. J. Vet. Res.* **50** 915–918 (1989)
- (18) SANCHEZ, M.S., FORD C.W., YANCEY R.J. JR.: *J. Dairy Sci.* **77** 1251–1258 (1994)
- (19) SAS: *SAS/STAT User's Guide*, release 6.11. SAS Inst. Inc., Cary, NC. (1995)
- (20) SHUSTER, D.E., KEHRLI, M.E. JR, STEVENS, M.G.: *Am. J. Vet. Res.* **54** 80–85 (1993)
- (21) SORDILLO, L.M., SHAFER-WEAVER, K., DEROSA, D.: *J. Dairy Sci.* **80** 1851–1865 (1997)
- (22) VAN KAMPEN, C., MALLARD, B.A., WILKIE, B.N.: *Vet. Immunol. Immunopathol.* **69** 23–32 (1999)
- (23) VILCEK, J., LEE, T.H.: *J. Biol. Chem.* **266** 7313–7316 (1991)
- (24) WERLING, D., SUTTER F., ARNOLD, M., KUN, G., TOOTEN, P.C., GRUYS, E., KREUZER, M., LANGHANS, W.: *Res. Vet. Sci.* **61** 252–257 (1996)
- (25) ZECCONI, A., BRONZO, V., HAMANN, J., CASULA, A.: In *Proc. 3rd IDF Intern. Mastitis Sem.* (Eds. A. Saran, S. Soback) Tel-Aviv, Israel, 28–32 (1995)
- (26) ZIA, S., GIRI, S.N., CULLOR, J., EMAU, P., OSBURN, B.I., BUSHNELL, R.B.: *Am. J. Vet. Res.* **48** 1617–162 (1987)