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Original article

Bovine blood neutrophil acyloxyacyl hydrolase (AOAH) activity during endotoxin and coliform mastitis

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Abstract – The dynamics of blood neutrophil acyloxyacyl hydrolase (AOAH) activity, the appearance of endotoxin (lipopolysaccharide, LPS) in blood and the role of blood neutrophil AOAH in the severity of *Escherichia coli* and endotoxin mastitis were investigated in early postpartum dairy cows experimentally challenged with either endotoxin (n = 6) or *E. coli* (n = 6). The AOAH activity of blood neutrophils started to decrease significantly at post challenge hours (PCH) 6–24 and 12–24 in the endotoxin and *E. coli*-challenged groups, respectively; it returned to pre-challenged values at PCH 48 in both endotoxin- and *E. coli*-challenged groups. The cows were classified as moderate and severe responders according to milk production loss in the non-challenged quarters at PCH 48. There were no severe responders in the endotoxin-challenged group. In the *E. coli*-challenged group, only 1 severe responder was identified. The pre-challenge neutrophil AOAH activity of the severe responder was ~30% lower than that of moderate responders. No LPS was detected in the plasma of endotoxin-challenged cows; neither was it found in the plasma of moderate responders in the *E. coli*-challenged group at any PCH. However, at PCH 6, a remarkable amount of LPS was detected in the plasma of the severe responder from the *E. coli*-challenged group. Furthermore, neutrophil AOAH activity was increased by ~70% in the severe responder at PCH 6, but it increased by only ~15% in moderate responders. This was followed by a decreased neutrophil AOAH activity at PCH 12–24 and 24–72 in moderate and severe responders, respectively; the decreased AOAH activity at those PCH was more pronounced in the severe responder. The pronounced decreased neutrophil AOAH activity during mastitis often coincided with extreme leukopenia, neutropenia and a maximal number of immature neutrophils in the blood. Our results demonstrate that a decrease in neutrophil AOAH activity results in the appearance of LPS in the blood, and low blood neutrophil deacylation activity could be considered as a risk factor for severe clinical coliform mastitis.

acyloxyacyl hydrolase (AOAH) / blood / endotoxin / mastitis / neutrophils

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1. INTRODUCTION

An invading pathogen must be held in check by the innate immune system until a specific immune response can be mounted. In the case of Gram negative bacteria, the principal stimulator of the innate immune system is lipopolysaccharide (LPS) or endotoxin, a compound of the bacterial outer membrane. In the udder, this LPS evokes several functional responses in the neutrophils that contribute to the innate immunity of the udder. The recruitment and activation of these short-lived, bone marrow-derived cells to the site of inflammation or infection are pivotal to limit the severity of mastitis [2, 3, 20, 22, 28, 30]. Around calving, the risk for severe clinical mastitis is extremely high; the main underlying reason for this high risk can be impaired blood neutrophil function [3,22,25].

Although LPS has been recognized as an important mediator of local and systemic symptoms during coliform and endotoxin mastitis [17, 20], the role of blood neutrophils in defense against LPS during *Escherichia coli* mastitis is not completely understood. While the presence of LPS in plasma is only short-lasting, its resorption from the mammary gland into the blood stream is possible during *E. coli* mastitis due to increased permeability of the blood-milk barrier; this triggers a marked increase of tumor necrosis factor alpha (TNF-α) in the blood. These events are restricted to severely diseased cows [1, 8, 20–22]. Severity of *E. coli* mastitis seems to be related to the enhanced release of secondary induced inflammatory mediators such as TNF-α [1, 14], which could result from impaired LPS detoxification in the blood.

There are many-known and unknown mechanisms, enzymatic and non-enzymatic, that neutralize LPS in the blood [15, 16, 18, 19]. Neutrophil granules contain an array of anti-LPS defense proteins such as lactoferrin, lysozyme and bactericidal-permeability increasing protein [15, 16, 25]. The 2-subunit lipase, acyloxyacyl hydrolase (AOAH), which is also present in bovine neutrophil granules [18, 19], hydrolyses and removes the secondary acyl chains of Gram-negative bacterial lipid A of endotoxin [12]; this results in a substantial decreased toxicity of LPS while retaining much of the immunostimulatory potency of native LPS [23]. Blood neutrophil AOAH activity has been found to be decreased in postpartum cows; this decrease was particularly pronounced in some cows, whereas in other cows no changes were observed [8]. Thus, in addition to other antibacterial or anti-LPS defense mechanisms such as the production of reactive oxygen species (ROS) [14, 20, 22], decreased AOAH activity could represent a risk factor for severe clinical coliform mastitis.

Indeed, since LPS is considered as a toxic compound to trigger a massive TNF-α release [1], and endotoxin shock, decreased LPS detoxification could play a critical role in the outcome of coliform mastitis. To date, no one has demonstrated the changes in neutrophil AOAH activity and plasma LPS concentrations during coliform/endotoxin mastitis. Furthermore, the role of neutrophil AOAH activity as a risk factor for the severity of *E. coli* mastitis in early postpartum dairy cows has not been investigated so far. Therefore, the objectives of the present study were the following: (1) investigate the temporal changes in neutrophil AOAH activity following exposure of the mammary gland to LPS and *E. coli*; (2) detect LPS in the blood; and (3) relate this with the occurrence of endotoxemia in cattle.

2. MATERIALS AND METHODS

This experiment was approved by the Ethics Committee of Ghent University, Faculty of Veterinary Medicine (Belgium).

2.1. Experimental animals

In total 12 healthy Holstein-Friesian cows in their first or second lactation and between 2 and 6 weeks after parturition were used as experimental animals. The animals, on a zero-grazing system from arrival till the end of the experiment, were put into an individual stall and were fed with a special ration for pregnancy and lactation. The cows had calved normally and showed no signs of periparturient diseases. They had free access to water and hay. After gestation, clinically healthy cows showing no signs of typical periparturient diseases were selected on the basis of 2 consecutive bacteriological negative milk samples and a milk somatic cell count (SCC) of $< 2.10^5$/mL milk per individual quarter. One week before the start of the experiment the animals were fed a daily ration of approximately 8 kg of concentrate and had free access to water and hay. They were milked twice daily at 8 a.m. and 5 p.m. with a 4-quarter milking machine (Packo & Fullwood, Zeddelgem, Belgium).

2.2. Induction of mastitis

In the first study, mastitis was experimentally induced by LPS infusion ($n = 6$) and in the second study, mastitis was induced by infusion of living *E. coli* bacteria ($n = 6$). In the first study, 10 mg LPS from *E. coli* O111:B4 (Sigma Chemical Co., St. Louis, MO, USA) was diluted in 100 mL pyrogen-free (9 g/L) saline solution and aliquoted in bottles of 5 mL LPS solution (500 μg in 5 mL). All air was removed from the bottles by a sterile N_2–flow. LPS solutions were stored at −20 °C until use. Frozen LPS solutions were thawed immediately before a challenge experiment and 15 mL of pyrogen-free saline solution was added. Before LPS injection the teat ends were disinfected with ethanol (70%) and mixed with chlorhexidine as previously described [20]. Endotoxin mastitis was induced – after the morning milking – into the left front and hind quarters by a single intramammary (i.mam) injection of 20 mL LPS solution per quarter (25 μg LPS/mL), final concentration) using a sterile teat cannula. After injection, each quarter was massaged for 30 s to distribute the LPS solution in the mammary gland.

In the second study, one hour after the morning milking, 10^4 cfu of *E. coli* P4:O32 (H37, ß-glucuronidase +, haemolysin −) were intramammarily injected into the 2 left udder quarters of each cow. Prior to inoculation, the bacteria had been cultured in Brain Heart Infusion broth (DIFCO Laboratories, Detroit, MI) for 18 h at 37 °C, washed, resuspended and diluted with sterile 0.01 M phosphate-buffered saline solution (PBS) as previously described [21,22]. After cleaning of the udder and sterilization of the teat ends, a total volume of 10 mL containing 10^4 *E. coli*/mL was injected into the left quarters using a sterile teat cannula; to better observe the signs of clinical mastitis, two quarters were used for the challenge. After injection of the *E. coli* suspension, each quarter was massaged for 30 s to distribute the bacteria in the quarters.

2.3. Blood sampling

Blood samples for leukocyte counting, differentiation, enumeration and isolation of neutrophils and determination of neutrophil AOAH activity were collected at post challenge hours (PCH) 0, 6, 12, 24, 48, 72, 144 and 216 after endotoxin and *E. coli* challenge. A total of 40 mL blood was collected at each sampling by venipuncture in Vacuette tubes (Greiner®, 9 mL) containing lithium heparin and stored on ice until processed. For LPS quantification in plasma, additional samplings were performed at

those PCH. To minimize exogenous LPS contamination, samples were collected in a pyrogen-free manner by puncture of the external jugular vein with disposable needles mounted on disposable tubes (Vacutainer®, Terumo, Belgium); endotoxin-free vacuum blood collection tubes containing 120 i.u. sodium heparin (Chromogenix AB, Mölndal, Sweden) were used. Plasma samples were frozen at −20 °C and stored until use for LPS quantification.

2.4. Classification of mastitis challenged cows into severe and moderate responders

Milk production (MP) of individual quarters was measured using a 4-quarter milking machine in order to determine MP in challenged and non-challenged quarters. The MP data from evening and morning milk were pooled to obtain the daily MP. The MP of non-challenged quarters at 2 days after challenge, compared with pre-challenge MP, was used for classification of mastitis challenged cows into severe (S, MP < 50% of pre-challenge MP) and moderate (M, MP > 50% of pre-challenge MP) responders according to [7, 13, 22].

2.5. Blood leukocyte enumeration, neutrophil isolation, preparation and differentiation

The number of leukocytes in whole blood was determined using an electronic particle counter (Coulter counter Z2, Coulter Electronics, Hialeah, FL, USA) after addition of 200 μL of Zap-Oglobin II lytic reagent (Beckman Coulter, Paris, France) to 10^5-fold diluted blood samples and gentle mixing. Neutrophils were isolated from whole blood as previously described [4, 20–22]. Double distilled water and buffers used for the isolation procedure were determined to be pyrogen-free using a qualitative limulus amebocyte lysate assay (BioWhittaker Inc., Walkersville, MD, USA). The final pellet of neutrophils was resuspended in 2.5 mL PBS (0.01 M). The total number of leukocytes in whole blood and isolated blood cells was determined using an electronic particle counter [20, 22]. The total number of different circulating leukocytes was determined by combination of the total number of blood leukocytes and the differentiation on smear preparations of blood samples [20, 22]. Differential cell counts and staining procedures were performed on whole blood similar to the isolates on eosin-Giemsa-stained (Merck Diagnostica, Darmstadt, Germany) smears, using light microscopy (Nikon labophot-2) at magnification ×1000. Cell identification was based on morphological characteristics as previously described [20, 22].

To quantify percentages of each cell type in the samples, a total of 200 cells per slide were classified as neutrophils (mature and immature), monocytes, lymphocytes and eosinophils. The total number of cells isolated was determined by using the cell counter, and the percentage of neutrophils was determined by direct examination. The isolation technique routinely provided large numbers of neutrophils that were determined to be viable by the propidium iodide exclusion method [21] and greater than 95% neutrophils in content. To prepare neutrophil lysates containing the AOAH enzyme, the isolated neutrophils were lysed with a buffer (100 mM KCl; 3.9 mM NaCl; 3.5 mM $MgCl_2$; 10 mM HEPES (N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid) (pH 7.4) that contained 1% (vol/vol) Nonidet P-40, 15 mM EDTA, and 75 g of phenylmethylsulfonyl fluoride per mL). The lysis buffer was added (1 mL.10^{-7} viable neutrophils), and the cells were incubated at room temperature for 10 min with frequent mixing; then the mixtures were centrifuged at

$2000 \times g$ for 10 min. Supernatant aliquots were stored at −70 °C, for quantification of neutrophil AOAH.

2.6. Detection of SCC and CFU in challenged quarters

To examine the dynamics of SCC and CFU in challenged quarters and to relate these changes to our main findings, milk samples were taken at PCH 0, 6, 12, 24, 48, 72, 144 and 216 of the endotoxin and *E. coli* challenge. The SCC was determined by a Fluoro-optoelectronic cell counting procedure (Fossomatic® 360; Foss Electronic, Eden Prairie, MN, USA). The maximal detection capacity of the method for SCC values was 10^7/mL. The CFU was performed throughout the study, using bacterial plate culture, as previously described [21, 22].

2.7. Neutrophil acyloxyacyl hydrolase (AOAH) activity

AOAH activity in neutrophil lysates was determined according to McDermott et al. [18, 19], modified by Dosogne et al. [8]. ^{3}H and ^{14}C radioactivity were measured simultaneously with a liquid scintillation counter (1219 Rackbeta, LKB Wallac). AOAH activity was measured as the radioactivity of ^{3}H-labeled fatty acids released from LPS whereas ^{14}C was used for correction of LPS backbone contamination of the fatty acid extracts. AOAH activity was expressed as picomole (pM) fatty acid released per 10^7 neutrophils per hour. All AOAH determinations were performed in duplicate.

2.8. Determination of LPS in plasma

The amount of LPS in plasma was determined using a quantitative limulus amœbocyte lysate (LAL) test (Biowhittaker Inc, Walkersville, Maryland, USA) according to Dosogne et al. [9]. Briefly, one endotoxin unit (EU) per mL platelet-rich plasma (PRP) corresponds to 100 pg LPS/mL PRP. The LPS detection limit of the assay for bovine plasma was 0.036 EU/mL PRP. The LPS values were only considered positive when it was higher than 2 times the detection limit; that is, > 0.072 EU/mL PRP or > 7.2 pg/mL LPS. The results are expressed in pg/mL.

2.9. Statistics

Statistical analysis of the data was performed using a mixed model with the cow as the random effect, time as a categorical fixed effect and milk reduction and its interaction with time as continuous fixed effects. Significance of the differences was determined at $P < 0.05^*$, $P < 0.01^{**}$ and $P < 0.001^{***}$.

3. RESULTS

3.1. Clinical findings and identification of severe (S) and moderate (M) responders

Intramammary *E. coli* and LPS provoked clinical signs (both local and systemic) of acute mastitis such as fever, tachycardia, decreased rumen motility and increase of CFU and SCC with decreased MP in all quarters of the cows. The MP in challenged quarters substantially decreased; even at PCH 12–54 it hardly yielded 100 mL for the experiment (data not shown). This decrease resulted from the damage to the gland caused by both systemic and local effects of mastitis. Most clinical signs peaked at PCH 6–24 and almost completely restored at PCH > 72 (Tab. I).

There were no S responders when using MP loss of non-challenged quarters

Table I. Comparison of some circulating leukocytes (WBC), mature neutrophils, immature neutrophils (sum of myelocytes and metamyelocytes) and AOAH (acyloxyacyl hydrolase) activity (pM f.a./10^6 neutrophils/h) of blood neutrophils and appearance of LPS in serum as well as the SCC and cfu dynamics in inflamed quarters of cows experimentally challenged with LPS ($n = 6$) and *E. coli* (moderate responders; $n = 5$, and severe responder; $n = 1$). ND = not detected; PCH = post challenge hours; time 0 = hour of mastitis induction. The values are means ± SEM. The significance of the difference between pre and post-infusion of LPS/*E. coli* is indicated with asterisks (*$P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

PCH	Parameter	Endotoxin mastitis group ($n = 6$)	*E. coli* mastitis group	
			Moderate responders ($n = 5$)	Severe responder ($n = 1$)
0	WBC/μL	7314 ± 213	9633 ± 658	8540
	Mature neutrophils /μL	1903 ± 80	2886 ± 290	1708
	Immature neutrophils /μL	216 ± 34	467 ± 97	256
	LPS in Serum (pg/mL)	ND	ND	ND
	AOAH activity	73.0 ± 2.8	70.8 ± 2.5	56.7
	SCC/mL (×1000)	81 ± 17	86.4 ± 6.1	71
	CFU/mL (×1000)	0	0	0
6	WBC/μL	2368 ± 299***	5974 ± 160***	6650
	Mature neutrophils /μL	454 ± 70***	1611 ± 30***	133
	Immature neutrophils /μL	554 ± 79**	636 ± 67*	532
	LPS in Serum (pg/mL)	ND	ND	26
	AOAH activity	47.9 ± 2.0**	81.0 ± 7.7*	92.9
	SCC/mL (×1000)	4516 ± 657	3900 ± 472	110
	CFU/mL (×1000)	0	14.1 ± 11.2	390
12	WBC/μL	4442 ± 1180**	2783 ± 373**	8160
	Mature neutrophils /μL	568 ± 141***	590 ± 85***	82
	Immature neutrophils /μL	868 ± 232**	400 ± 48	979
	LPS in Serum (pg/mL)	ND	ND	93
	AOAH activity	59.8 ± 2.1**	48.6 ± 4.1***	58.6
	SCC/mL (×1000)	> 10000	7680 ± 674	4900
	CFU/mL (×1000)	0	25.3 ± 9.4	1400
24	WBC/μL	12372 ± 2125**	5182 ± 389**	3340
	Mature neutrophils /μL	1965 ± 420	988 ± 110**	67
	Immature neutrophils /μL	2030 ± 411**	1138 ± 137	1536
	LPS in Serum (pg/mL)	ND	ND	9.7
	AOAH activity	65.4 ± 1.7*	55.4 ± 8.5***	24.6
	SCC/mL (×1000)	> 10000	4800 ± 424	> 10000
	CFU/mL (×1000)	0	8.9 ± 4.68	420
48	WBC/μL	12222 ± 1244**	8944 ± 236	2580
	Mature neutrophils /μL	1967 ± 284	1629 ± 100*	26
	Immature neutrophils /μL	1283 ± 103**	1495 ± 168**	1367
	LPS in Serum (pg/mL)	ND	ND	ND
	AOAH activity	70.0 ± 1.5	72.4 ± 1.2	18.2
	SCC/mL (×1000)	3390 ± 613	2080 ± 305	> 10000
	CFU/mL (×1000)	0	1.9 ± 0.62	180

Table I. Continued.

PCH	Parameter	Endotoxin mastitis group (n = 6)	*E. coli* mastitis group	
			Moderate responders (n = 5)	Severe responder (n = 1)
	WBC/μL	8155 ± 1656	11315 ± 464**	3560
	Mature neutrophils /μL	1360 ± 305*	2014 ± 248	36
	Immature neutrophils /μL	512 ± 119*	1349 ± 167**	2100
72	LPS in Serum (pg/mL)	ND	ND	ND
	AOAH activity	72.0 ± 1.6	96.6 ± 5.9**	14.8
	SCC/mL (×1000)	1127 ± 167	867 ± 297	7950
	CFU/mL (×1000)	0	0.45 ± 0.2	35
	WBC/μL	10230 ± 1317*	11352 ± 475**	7110
	Mature neutrophils /μL	1735 ± 214	1902 ± 128	995
	Immature neutrophils /μL	430 ± 44*	1226 ± 107**	1635
144	LPS in Serum (pg/mL)	ND	ND	ND
	AOAH activity	74.3 ± 1.4	70.7 ± 4.1	68.7
	SCC/mL (×1000)	217 ± 9	274 ± 39	5500
	CFU/mL (×1000)	0	0	5.5
	WBC/μL	9055 ± 246*	10462 ± 565*	8640
	Mature neutrophils/μL	1808 ± 69	1956 ± 159	1123
	Immature neutrophils/μL	257 ± 30	818 ± 84*	1642
216	LPS in Serum (pg/mL)	ND	ND	ND
	AOAH activity	68.2 ± 1.4	77.2 ± 3.2	69.2
	SCC/mL (×1000)	107 ± 12	112 ± 15	4100
	CFU/mL (×1000)	0	0	5.6

at PCH 48 in the endotoxin-challenged group. In the *E. coli*-challenged group, 1 cow was identified as an S responder, whereas the other 5 cows in the *E. coli* model were M responders. The MP of non-challenged quarters was decreased to 5% of the pre-challenge MP in the S cow and extremely low until 5 days after challenge (Fig. 1). In M cows, the MP of non-challenged quarters 2 days after challenge was almost the same volume as the pre-challenge MP and was completely recovered at 3 days after challenge.

3.2. Blood leukocyte enumeration, differentiation and neutrophil immaturity

After challenge, both in endotoxin and *E. coli* challenged cows, a transient leukopenia, leukocytosis, neutropenia and neutrophilia was observed (Tab. I). Significant leukopenia was observed between PCH 6 and 12 and 6–24 in endotoxin and *E. coli* mastitis, respectively, regaining, even exceeding, pre-challenge values after those PCH (Tab. I). Compared to pre-challenged values, the number of circulating mature neutrophils was significantly ($P < 0.001$) minimal at PCH 6 and 12 in endotoxin and *E. coli* mastitis groups, and in *E. coli* it still remained significantly low till PCH 48. This decrease often coincided with an increased number of immature neutrophils (metamyelocyte and myelocytes). Blood neutrophil numbers changed significantly over time ($P < 0.0001$). Blood neutrophil numbers decreased significantly with increasing milk reduction ($P = 0.050$). In the S responder with the highest reduction

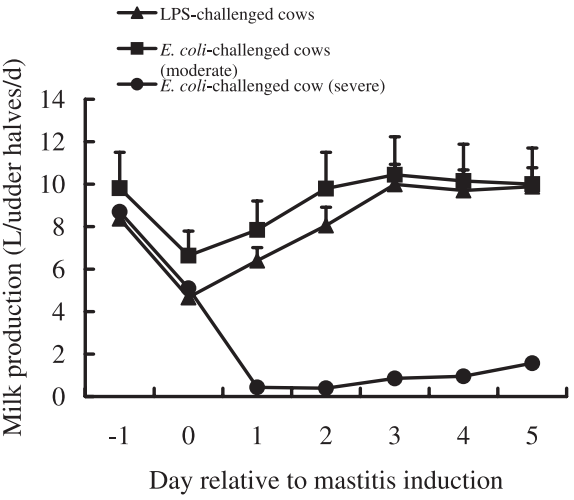

Figure 1. Daily milk production (liters/udder halves) from the non-challenged contralateral quarters from LPS-challenged cows (n = 6 ± SEM; triangles), *E. coli*-challenged cows (moderate; n = 5 ± SEM; squares) and *E. coli*-challenged cows (severe; n = 1; circles) during mastitis. Day 0 = day of mastitis induction.

the decrease was even reached to 133 neutrophils/μL at PCH 6. The reduction in blood neutrophil numbers of this cow was severe and remained extremely low between PCH 6 and 72 (less than 5% of pre-challenged values; Tab. I).

Mature blood neutrophil numbers changed significantly over time ($P < 0.0001$). Mature blood neutrophil numbers decreased significantly with increasing milk reduction ($P = 0.002$). The pattern of decrease in immature neutrophils (metamyelocyte and myelocytes) was remarkable in the S responder (Tab. I).

3.3. SCC and CFU in challenged quarters

The overall pre-challenged SCC was the same for endotoxin, M and S groups. In endotoxin challenged quarters, at PCH 12–24 the SCC value exceeded the maximal detection capacity of the method (Tab. I). The SCC in endotoxin and M groups, however, increased faster (maximal at PCH 12). In the S cow the maximal SCC appeared much later (at PCH 24). Throughout the experiment the SCC values did not reach the normal in the S cow (see Tab. I).

In all groups before the experiment, and in the endotoxin group throughout the experiment, the CFU was always zero. The CFU in milk of the S cow increased much faster (at PCH 6) and did not reach zero throughout the infection, whereas in M cows it was not substantially increased and it reached zero at PCH 144 and onwards (Tab. I).

3.4. Blood neutrophil acyloxyacyl hydrolase activity

Although both groups were moderate responders, the pattern of changes in neutrophil AOAH activity in the endotoxin-challenged group was different from those of the *E. coli*-challenged (the M) group (Tab. I, Fig. 2). After challenge, in the *E. coli* (M) group, AOAH activity increased (at PCH 6; $P < 0.05$), then remarkably decreased (at PCH 12–24; $P < 0.001$), reaching pre-challenged values at PCH 48 and again increased (at PCH 72; $P < 0.001$), and finally reached the pre-challenged values; whereas in the endotoxin group, the neutrophil AOAH activity first decreased (at PCH 6–24; $P < 0.01$) then reached the pre-challenged values at

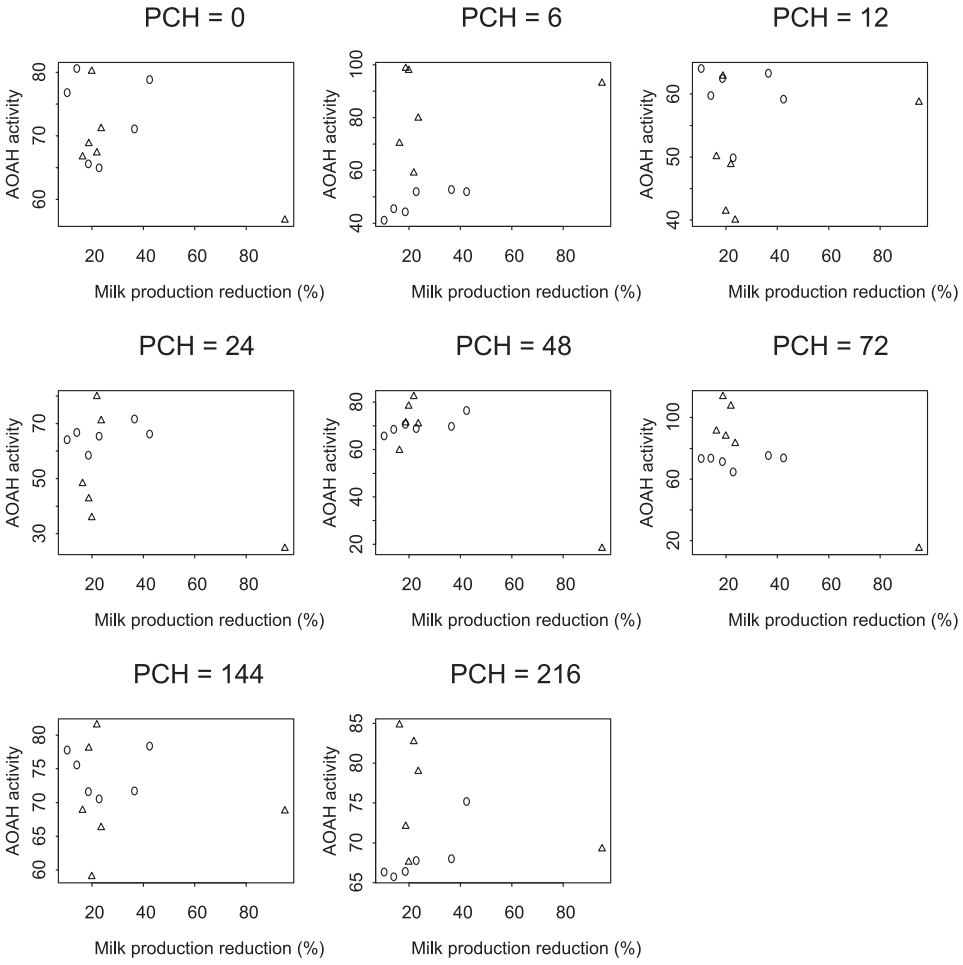

Figure 2. Changes in AOAH activity (pM f.a./10^6 neutrophils/h) of blood neutrophils and its relation with milk production reduction of non-challenged quarters at 2 days after challenge during experimentally induced endotoxin mastitis (n = 6; circles) and *E. coli* mastitis (n = 6; triangles); PCH = post challenge hours. The triangle, which is located at the most right part of the figures, represents the values for the severe responder.

PCH 48 and onwards (Tab. I, Fig. 2). Therefore, a rebound effect was observed at PCH 72 only in M cows after re-establishment of blood neutrophil AOAH activity at PCH 48.

Neutrophil AOAH activity changed significantly during infection ($P < 0.0001$). On average neutrophil AOAH activity decreased significantly with increasing milk reduction ($P = 0.0004$). There was, however, also a significant interaction between time and milk reduction ($P < 0.0001$). This was due to the fact that the neutrophil AOAH activity of the severe responder increased dramatically at PCH 6, reaching pre-challenged values and again hugely decreased (Tab. I), which was not the case for the endotoxin and M groups.

Milk reduction increased with lower value of pre-challenged neutrophil AOAH activity; indeed, before challenge, the neutrophil AOAH activity of the S responder was ~30% lower than that of M responders (Tab. I). At PCH 6, neutrophil AOAH activity was increased by ~70% in the S responder, which was remarkably different from those of other groups. Overall, throughout the study, the changes on AOAH activity was the most pronounced in the severe responder (Tab. I, Fig. 2).

3.5. LPS in plasma

In endotoxin-challenged cows as well as in the *E. coli* challenged cows (M group), no LPS was detected in the plasma at any time point following intramammary challenge. A remarkable amount of LPS (26, 93 and 9.7 pg/mL at PCH 6, 12 and 24, respectively) was detected in the plasma (Tab. I) but only in the S cow of the *E. coli* challenged group.

4. DISCUSSION

The major goal of our experiment was to examine the temporal changes on neutrophil deacylation activity and serum LPS concentration during both endotoxin and *E. coli* mastitis. The fluctuations in blood neutrophil AOAH activity during both endotoxin and *E. coli* mastitis were consistent with other findings [18, 19]. This study shows, however, a novel-detailed information on temporal changes, especially during the critically earliest stages of mastitis, during which the nature of the cow's innate immune response governs whether and how to eliminate an intramammary infection from the gland [3, 22, 28]. In addition, we distinguished between severely and moderately diseased cows based on the MP loss in non-challenged quarters. The MP loss in non-challenged quarters has been shown to be an appropriate parameter to estimate systemic illness during *E. coli* mastitis [2, 7, 13, 21, 22].

Many host-derived cytokines such as interleukin-1 (IL-1), IL-6 [25, 26] and TNF-α [14] are produced during endotoxin/coliform mastitis; they cause systemic effects such as fever and increase the bone marrow output of leukocytes to compensate for the decrease in the circulating pool [20–22]. Consequently, there is a transient increase in the number of circulating immature neutrophils, increased permeability of capillaries and the blood/milk barrier, and a concomitant exudation of some blood proteins in the mammary gland [14, 21]. Increased permeability of the blood/milk barrier was indicated by the appearance of clots in milk at PCH 6–24. Between PCH 6 and 24, the influx of a large number of neutrophils in the challenged quarters resulted in a substantial increase of the SCC (see Tab. I). The recruitment of neutrophils into the challenged quarters, as reflected by the SCC, together with their increased functionality [20–22] are both important aspects of the defense mechanism against *E. coli*/endotoxin during coliform mastitis. In this study the two left quarters were injected in order to better assess the signs of clinical mastitis, and the cows' responses to the endotoxin/*E. coli* challenge would have been less intensive if we had used only one quarter.

The increased milk SCC in challenged quarters coincided with decreased CFU. Although no significant differences on pre-challenged SCC were observed between all groups, the SCC increased much faster in endotoxin and M groups (see Tab. I). This is a classic immunological phenomenon [3, 21, 22, 25] that was more appropriate in endotoxin and M groups, substantially lessening CFU (Tab. I). The reason why a remarkable amount of LPS was detected at PCH 6–12 would be that at these PCH there were very low levels of mature neutrophils, and that an array

of LPS-neutralizing proteins (e.g., cationic peptides) produced by neutrophils may be at low levels due to low numbers of mature neutrophils (see Tab. I, Fig. 2). Furthermore, at those PCH the extremely rapid/unstoppable increase of CFU (*E. coli* growth) resulted in a huge imbalance between AOAH and its substrate (LPS) in the S cow, and the body's AOAH capacity was insufficient to deacylate LPS. Therefore, the initial *E. coli* growth may result in an extremely high LPS production and AOAH-LPS deacylation imbalance. This might result from a far slower increase of SCC in the gland at those PCH (see Tab. I). This exciting topic needs to be further studied.

Local signs of mastitis such as swelling and pain of the challenged quarters, appearance of flecks and milk leakage in challenged quarters were observed at PCH 6 to 24 (data not shown) for all groups. These local signs were more pronounced and prolonged in the S cow, exactly indicating a greater breakdown of the blood-milk barrier in the severe responder, potentially enhancing efflux of LPS to the blood. However, at PCH 48–78, during which the AOAH activity and mature neutrophils were the lowest in this cow, we expected to have detectable LPS, but the LPS was not detected in the blood. The most acceptable reason for these disparities would be the "non-enzymatic substances" in mastitis milk and serum that might contribute to endotoxin neutralization [18, 19, 22]. For example, inhibition of neutrophil AOAH activity during natural cases of mastitis caused by both Gram negative and Gram positive organisms [18, 19] supports the involvement of non-enzymatic local-and-systemic mediators like LPS binding proteins (LBP), lactoferrin, TNF-α [1, 32] in LPS neutralization. Although the LPS molecule is not degraded by, for instance, LBP, the LPS-LBP bond could result in decreased bioavailability of the LPS molecule, thereby reducing the toxic effects of endotoxin during *E. coli*/endotoxin mastitis. The underlying mechanism of these differences remains to be investigated.

Accordingly [8, 9, 18, 19], before challenge and during early hours of mastitis, the major source of LPS neutralizing factors are predominantly neutrophil AOAH. This might be a decisive factor in deacylation and thereby detoxification of LPS thus prevents prolonged mastitis. Interestingly, before challenge, blood neutrophil AOAH activity was lower in S than in M cows. Our data, therefore, suggest decreased LPS detoxification mechanisms. Indeed, in the cow with impaired blood neutrophil AOAH activity before challenge, MP was strongly decreased for several days following intramammary *E. coli* challenge and LPS were detected in plasma, whereas in the other cows MP was restored very rapidly and no LPS were found in plasma. In addition to an impairment of blood neutrophil AOAH activity, the number of blood neutrophil was also drastically decreased in the S cow (see Tab. I), suggesting a further decrease of total LPS detoxification capacity in the blood. The results from our study agreed with other studies [8, 13, 22, 26, 30] suggesting that the occurrence of LPS in plasma of severely diseased cows could be the result of a decreased detoxification capacity of blood neutrophils. In our study, it was possible that in M cows the *E. coli* endotoxins were effectively controlled by milk neutrophil AOAH, though milk neutrophil AOAH activity was not assessed.

The AOAH activity per neutrophil in endotoxin mastitis was also changed, however, the time of the alteration was different from that of the *E. coli* challenged group (see Tab. I, Fig. 2). The rapid decreased neutrophil AOAH activity after LPS challenge in comparison with *E. coli* challenge might be due to a faster inflammatory response caused by intramammary administration of LPS. In contrast, intravenous

administration of LPS to rabbits resulted in a rapid (within 90 min) increase of plasma AOAH activity [10]. So, a different route of LPS administration leads to different temporal changes in neutrophil AOAH activity. Surprisingly, intramammary administration of *E. coli* resulted in faster increased AOAH activity of neutrophils (PCH 6). Therefore, it is possible that, in contrast to endotoxin, live bacteria elicit an increase in AOAH activity in both M and S responders immediately after infection. This might be due to the involvement of a phagocytosis phenomenon even by mammary epithelia during maximal immune response which then releases *E. coli* in the tissue, intact [6]. In the LPS model this phenomenon is almost non-existent.

In our study, blood neutrophil AOAH activity was decreased at PCH 12–24 and 6, 24–72 in M and S groups, respectively, with a much more pronounced effect in the S cow. This has not been previously reported. These novel findings could be explained by neutrophil immaturity, since neutrophil granule enzymes are acquired along their maturation pathway [11, 29]. The release of immature neutrophils during *E. coli* mastitis was consistent with previous reports [13, 21, 22, 30] and can be ascribed to indirect hematopoietic effects of LPS on the bone marrow. The long-lasting impairment of blood neutrophil AOAH activity in the S cow could be explained by its prolonged shift to the left (intense production of immature neutrophils; see Tab. I).

Together, our findings suggest a direct or indirect stimulatory effect of LPS on blood neutrophil AOAH activity during *E. coli* mastitis. In the S cow, intense stimulatory effects were observed very early (PCH 6), whereas in M cows, despite a mild increase at PCH 6, an intense increase was at a later stage of the inflammation (PCH 72); this could be due to rapid LPS resorption and increased neutrophil immaturity in the S cow. The rebound effect of neutrophil AOAH activity in M cows was consistent with previous results [18, 19] and can be explained by the stimulatory effect of secondary induced inflammatory mediators. In a previous experiment, the priming effect for enhancement of neutrophil ROS production in M responders at PCH 72 was tentatively explained by the action of endogenous inflammatory mediators such as interferon-gamma but not LPS [13, 22], as in our study. Indeed, enhancement of neutrophil AOAH activity was not observed in the S cow at PCH 72, during which no LPS was detected in its plasma. Therefore, the impairment of neutrophil AOAH activity at those PCH can partly be ascribed to extreme neutropenia with increased neutrophil immaturity.

In this study only intracellular AOAH activity of neutrophils was investigated. Therefore, our results might explain the notion that LPS first had to be endocytosed by neutrophils in order for the intracellular AOAH-dependent detoxification (and other LPS detoxification mechanisms) to take place. It is generally accepted that LPB first forms a complex with LPS, which then binds to the CD14 molecule and TLR, especially TLR4, on phagocytes [27]. The β2-integrin CD11b/CD18 has also been described as an LPS receptor on bovine neutrophils [24]. Intramammary LPS infusion induced an upregulation of CD11b/CD18 and CD14 on neutrophils [5, 24, 30] for further LPS recognition. Extracellular AOAH activity was not measured in the present study. Because it has been demonstrated that extracellular and intracelluar AOAH activities of neutrophils show the same trend during induced *E. coli* mastitis [18, 19], it may be expected that extracellular AOAH activity also showed the same trends as intracellular neutrophil AOAH activity in our study.

The current study has at least demonstrated that a decreased intracellular neutrophil AOAH activity together with extreme leukopenia, neutropenia and a maximal number of immature neutrophils

was associated with the occurrence of LPS in plasma, indicating a significant role of not only the quantity of neutrophils, but also, as importantly, the quality of neutrophils (AOAH production capacity) during *E. coli* mastitis. Besides AOAH, bovine neutrophil granules also contain other LPS binding cationic proteins such as lactoferrin, and a wide variety of cationic peptides and oxidative enzymes that contribute to enhance neutrophil quality and LPS neutralization [15, 16, 21, 22, 31].

In short, this study demonstrates that a decrease in neutrophil AOAH activity results in the appearance of LPS in the blood, and low blood neutrophil deacylation activity could be considered as a risk factor for severe clinical coliform mastitis.

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