The Hsp72 response in peri-parturient dairy cows: relationships with metabolic and immunological parameters

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Abstract The study was aimed at assessing whether the peri-parturient period is associated with changes of intracellular and plasma inducible heat shock proteins (Hsp) 72 kDa molecular weight in dairy cows, and to establish possible relationships between Hsp72, metabolic, and immunological parameters subjected to changes around calving. The study was carried out on 35 healthy peri-parturient Holstein cows. Three, two, and one week before the expected calving, and 1, 2, 3, 4, and 5 weeks after calving, body conditions score (BCS) was measured and blood samples were collected to separate plasma and peripheral blood mononuclear cells (PBMC). Concentrations of Hsp72 in PBMC and plasma increased sharply after calving. In the post-calving period, BCS and plasma glucose declined, whereas plasma nonesterified fatty acids (NEFA) and tumor necrosis factor- α increased. The proliferative responses of PBMC to lipopolysaccharide (LPS) declined progressively after calving. The percentage of PBMC expressing CD14 receptors and Toll-like receptors (TLR)-4 increased and decreased in the early postpartum period, respectively. Correlation analysis revealed significant positive relationships between Hsp72 and NEFA, and between PBMC proliferation in response to LPS and the percentage of PBMC expressing TLR-4. Conversely, significant negative relation-

ships were found between LPS-triggered proliferation of PBMC and both intracellular and plasma Hsp72. Literature data and changes of metabolic and immunological parameters reported herein authorize a few interpretative hypotheses and encourage further studies aimed at assessing possible cause and effect relationships between changes of PBMC and circulating Hsp72, metabolic, and immune parameters in dairy cows.

Keywords Hsp72 · Peripheral blood mononuclear cells · Metabolism · Peri-partum · Dairy cows

Introduction

Heat shock proteins (Hsp) are known to be highly conserved and ubiquitous proteins synthesized in response to several stimuli (Liu and Steinacker 2001; Kregel 2002). In a recent review paper (Asea 2007), Hsp inducers have been categorized as environmental (heat shock, UV radiation, heavy metals, amino acids, oxidative stress, etc.), pathological (bacterial or parasitic infections, fever, inflammation, etc.), and physiological (growth factors, cell differentiation, hormonal stimulation or tissue development, caloric restriction, etc.).

Even if Hsp are typically regarded as intracellular proteins, in the last few years, several studies carried out in humans or laboratory animals demonstrated the implication of circulating inducible Hsp 72 kDa molecular weight (Hsp72) in a number of physiological or pathological events (Johnson and Fleshner 2006; Calderwood et al. 2007). Hsp are primarily released into blood during tissue injury, causing cell lysis or necrosis (Kimura et al. 2004; Lancaster and Febbraio 2005). However, other studies demonstrated that several cell types may exocytotically

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release Hsp72, and that elevation of circulating Hsp72 may be found also in response to stressors, which are unlikely to cause cell death (Fleshner and Johnson 2005; Johnson and Fleshner 2006).

Whether intracellular or plasma concentrations of Hsp72 are subjected to changes during the peri-parturient period has not been ascertained before either in cows or other species. However, the dramatic changes in the endocrine status due to the passage from pregnancy to lactation, calving itself, and the frequent occurrence during early lactation of metabolic and digestive disorders, oxidative stress, inflammatory dysfunctions, immunosuppression, environmental stress, and infections (Mulligan and Doherty 2008; Sordillo et al. 2009) suggest that some changes in the intracellular or circulating levels of these multifunctional proteins may occur in dairy cows around calving. Furthermore, literature data referred to other species also suggest that Hsp72 changes may have an adaptive significance in this intricate context (Asea et al. 2000; Fan and Cook 2004; Aneja et al. 2006; Chiu et al. 2009; Morino et al. 2008).

With regard to the immunomodulatory role of Hsp72, recent studies reported that this protein may modulate the immune response by binding selectively to a number of immune cells (Asea 2005). Hsp70 utilizes both Toll-like receptor (TLR)-2 (receptor for Gram-positive bacteria) and TLR-4 (receptor for Gram-negative bacteria) to transduce its proinflammatory signal in a CD14-dependent fashion (Asea et al. 2002; Chase et al. 2007).

Therefore, the present field study aimed at assessing whether the peri-parturient period is associated with changes of intracellular and plasma Hsp72 in dairy cows, and establishing possible relationships between Hsp72, and metabolic or immunological parameters subjected to changes around calving. Because of the well-known implication of Hsp in the physiology of heat stress, the study was carried out in two distinct groups of cows, which gave birth during thermoneutral spring or hot summer.

Materials and methods

Cows, measurements, and samplings

The study was carried out in a commercial herd on 35, healthy peri-parturient Holstein cows in the period March 31st–August 29th 2008. Seventeen cows gave birth in spring (SP), from April 14th to May 12th. The remaining 18 cows gave birth in summer (SU), between June 18th and July 19th. The two groups of cows were balanced for parity. In details, both groups had five first- and four second-calving cows; the remaining eight subjects in the SP groups were five third- and three fourth-calving cows, whereas the

remaining nine subjects in the SU group were four third- and five fourth-calving cows.

Values of maximum and minimum air temperature (°C) and relative humidity (%) were recorded daily by a weather station (Vantage-Pro 2 Plus, Davis Instruments, Hayward, CA, USA) installed in the proximity of the cows' building. Data of air temperature and relative humidity were utilized to calculate maximum and minimum temperature humidity index (THI) according to the Kelly and Bond formula (Kelly and Bond 1971), as extensively reported elsewhere (Vitali et al. 2009).

The body condition score (BCS) of cows was established at the beginning of the study, and then on a weekly basis using a five-point scale (ADAS 1986). Briefly, the system of body condition scoring is based on assigning a score to the amount of fat observed on several skeletal parts of the cow, and provides an indication of the energy status.

Three, two, and one week before the expected calving, and 1, 2, 3, 4, and 5 weeks after calving, venous blood was collected by jugular venipuncture using sodium heparin (10 IU/ml) as an anticoagulant. Immediately after collection, blood samples were stored in a portable refrigerator and transferred to the laboratory.

Laboratory analyses

Analysis of plasma

An aliquot of heparinized blood was centrifuged for 15 min at 3,500 rpm and the collected plasma was used to determine the concentrations of Hsp72, glucose, nonesterified fatty acids (NEFA), and tumor necrosis factor- α (TNF- α). Plasma Hsp72 concentrations were measured by a commercial ELISA kit according to the manufacturer's instructions (Stressgen, Bologna, Italy). The intra-assay coefficient of variation (CV) amounted to 2.7%. Plasma glucose and NEFA were measured by using commercial kits (Instrumentation Laboratory, Lexington, MA, USA, and Wako Fine Chemical Industries, Dallas, TX, USA, respectively).

TNF- α was determined by a cytotoxicity assay on WEHI 164 cells following the procedure described elsewhere (Asai et al. 1993). Cytokine concentrations in plasma samples were determined from a standard curve created with a reference preparation of human recombinant TNF- α (Pierce Endogen, Rockford, IL, USA). We wish to notice that plasma concentration of TNF- α was established by a bio-assay, which actually measures the plasma concentrations of bio-active cytokine beyond those of antagonist molecules (TNF- α soluble receptors and the like), thus providing an indication of the actual extent of the host's inflammatory response and of its fine tuning by homeostatic control factors.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as already described (Lacetera et al. 2001) with minor modifications. Briefly, blood diluted in phosphate-buffered saline (PBS, Sigma, Milano, Italy) was layered over Ficoll-Paque PLUS (APB, Milano, Italy) and centrifuged. The mononuclear cell band was recovered and washed twice in PBS. Residual red blood cells were eliminated by hypotonic shock treatment using redistilled water. PBMC recovery and viability were determined by a hemocytometer using the trypan blue exclusion method. Viability of PBMC typically exceeded 90%. An aliquot of PBMC was resuspended in RPMI 1640 medium (50%) + fetal bovine serum (FBS, 40%) + dymethylsulfoxide (10%) at a concentration of 4×10^6 viable cells/ml and stored at -80°C until analysis.

Remaining PBMC were resuspended at 1×10^6 viable cells/ml in RPMI 1640 medium containing 25 mM HEPES + 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B/ml (Sigma). The time between blood collection and establishment of cultures was always less than 6 h.

PBMC proliferation assay

One hundred microliters of PBMC were added into triplicate wells of 96-well flat-bottomed tissue culture plates. The T-lymphocyte mitogen Concanavalin A (ConA) (Sigma) was added at a final concentration of 2.5 $\mu\text{g}/\text{ml}$. The B-lymphocyte mitogen lipopolysaccharides (LPS) (from *Escherichia coli* 0111:B4, Sigma) was added at a final concentration of 20 $\mu\text{g}/\text{ml}$. Results of preliminary experiments indicated that these concentrations of mitogens induced optimal responses under the culture conditions under use. Control wells contained 100 μl of PBMC suspension (1×10^6 viable cells/ml) without mitogens (unstimulated). According to the guidelines provided by the manufacturer (APB), additional control wells contained 100 μl of complete RPMI-1640 or 100 μl of PBMC suspension without 5-bromo-2'-deoxyuridine (BrdU, see below). Tissue culture plates were thus incubated for 48 h at 39°C in an atmosphere of 95% air and 5% CO_2 . Thereafter, 100 μM of the pyrimidine analogue BrdU in 10 μl of RPMI-1640 medium was added to each well to give a final concentration of 10 μM BrdU. Following a further 18-h incubation, culture medium was removed by centrifuging the tissue culture plates at $300 \times g$ for 10 min. Thereafter, the tissue culture plates were dried at 60°C for 1 h. Cell proliferation was measured by an ELISA kit based on the measurements of BrdU incorporation during DNA synthesis in proliferating cells. Tests were performed

according to the guidelines provided by the manufacturer (APB). The incubation time with peroxidase-labelled monoclonal anti-BrdU antibody was 90 min. The absorbance values were read spectrophotometrically after a 25-min incubation with the substrate. Values for PBMC proliferation were expressed as optical density (OD) recorded at 450-nm wavelength in both unstimulated and mitogen-stimulated wells, minus the OD recorded in control wells without BrdU.

Further analysis on PBMC

Frozen PBMC was rapidly thawed at 38°C and concentrations of Hsp72 in PBMC were determined by an ELISA assay using a commercial kit according to the guidelines provided by the manufacturer (Stressgen). The intra-assay CV amounted to 1.3%.

Other aliquots of PBMC were thawed as above and submitted to flow cytometry (FC) analyses after two washings in FC buffer (PBS + 2% FBS + 0.1% Azide). To this purpose, PBMC were equally divided into three aliquots (1×10^6 cells each) and centrifuged again (2,000 rpm, 7 min, 5°C). One aliquot was stained (30 min at 4°C) with anti-human CD14-Phycoerythrin (PE) mAb (code MCA1568PE, Serotec, Kidlington, UK); the second one was stained with anti-human TLR-4-PE mAb (code MCA2061PE, Serotec) according to the manufacturer's directions; the third aliquot was kept under the same conditions as the unstained control. The use of the above anti-CD14 mAb with bovine PBMC has been already reported (Jacobsen et al. 1993). The anti-TLR-4 mAb under use has been previously shown to stain bovine PBMC in our lab (Amadori unpublished). After staining, cells were washed twice in FC buffer and resuspended in 300 μl of the same buffer. Samples were analyzed on a Bryte HS flow cytometer (BIO-RAD Microscience, Hemel Hempstead, UK) using Win-Bryte software. The typical forward and side scatter, lymphocyte/monocyte gate was set to exclude dead cells from the analysis. Ten thousand events per sample were acquired within this gate. The percentage of mAb-treated cells beyond the threshold FL2 fluorescence channel was assessed by subtracting the same percentage observed in control unstained cells.

Statistical analysis

Data of intracellular and plasma Hsp72, BCS, plasma glucose, NEFA and TNF- α , DNA synthesis in PBMC stimulated with mitogens, and percentages of PBMC expressing CD14 receptors, and TLR-4 were analyzed by a repeated measures analysis of variance (ANOVA) (StatSoft, 2004). First of all, we tested a model that included the effects of calving season, day relative to

calving, and cow. Results of this ANOVA indicated that calving season did not significantly affect the parameters under study, and none of the mean values of the parameters under study differed significantly between SP and SU cows. For this reason, we dropped calving season from the final model. Therefore, results reported below will be presented and discussed as referred to a single group of 35 peri-parturient dairy cows without calving season distinction. Multiple comparisons were carried out with Bonferroni post hoc test. Data were reported as least square means $\pm$ standard error (SE), and significance was declared at $P < 0.05$.

Correlation analysis was performed according to the method of Pearson using the same computer package. Significance was declared at $P < 0.05$.

Results

Daily values of maximum and minimum THI recorded during the study are reported in Fig. 1. As expected, SU cows were exposed to maximum and minimum THI higher than those experienced by SP cows. The mean values of maximum and minimum THI recorded during the two calving seasons were respectively 67.1 ± 5.6 and 48.5 ± 6.7 for spring and 78.8 ± 2.5 and 59.4 ± 3.2 for summer.

Figure 2 reports plasma (panel A) and PBMC (panel B) concentrations of Hsp72. In both cases, mean values significantly increased after calving. The highest concentrations of plasma Hsp72 were recorded at the end of the study period (35 days after calving), and values recorded on days 28 and 35 were higher ($P < 0.05$) than those recorded before. The highest values of intracellular Hsp72 were recorded on day 14 after calving, when mean values were

Fig. 1 Values of maximum (solid line) and minimum (dashed line) temperature humidity index (THI) during the study period. THI was calculated according to the Kelly and Bond formula (Kelly and Bond 1971) on the basis of maximum and minimum air temperature ($^{\circ}\text{C}$) and relative humidity (%), which were recorded daily by a weather station installed in the proximity of the cows' building

Fig. 2 Changes of inducible heat shock protein 72 kDa molecular weight (Hsp72) concentrations in plasma (a) and peripheral blood mononuclear cells (PBMC) (b) of peri-parturient dairy cows. PBMC and plasma were isolated from jugular blood of 35 Holstein cows, which were bled on a weekly basis during the last 3 weeks of pregnancy and the first 35 days of lactation. Both PBMC and plasma Hsp72 were measured by an ELISA system. Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P < 0.05$)

higher ($P < 0.05$) compared to those recorded before calving.

BCS changes are reported in Fig. 3. As expected, BCS started to decline after parturition, and the lowest values were recorded at the end of the study. After 21, 28, and 35 days of lactation, mean values of BCS were lower ($P < 0.05$) than those recorded before calving.

Plasma glucose declined after calving, and the lowest values were recorded on days 7 and 14 of lactation (Fig. 4). After 7 and 14 days of lactation, mean values of plasma glucose were lower ($P < 0.05$) with respect to values recorded at the beginning of the study (21 days before calving). Conversely, plasma NEFA increased in the post-calving period and peaked after 7 days of lactation (Fig. 5). At this time and after 14 days from calving, mean values of plasma NEFA were higher ($P < 0.05$) than those recorded before parturition. Plasma TNF-alpha increased after calving, even if only after 14 days of lactation mean values were higher ($P < 0.05$) compared to the pre-calving period (Fig. 6).

Fig. 3 Changes of body condition score (BCS) in peri-parturient dairy cows. The BCS was measured on a weekly basis in 35 Holstein cows during the last 3 weeks of pregnancy and the first 35 days of lactation. The BCS was established using a five-point scale (ADAS 1986). Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P<0.05$)

The proliferative response of PBMC to ConA did not change significantly during the study period (not shown). Conversely, the proliferative responses of PBMC to LPS declined progressively after calving and reached the lowest values at the end of the study (Fig. 7). After 21, 28, and 35 days of lactation, mean values of proliferative response (OD) were lower ($P<0.05$) with respect to values recorded at 21 and 14 days before calving.

The percentage of PBMC expressing CD14 receptors (mainly monocytes) increased in the early postpartum period, and the highest value was recorded at day 7 after calving, when mean values were higher ($P<0.05$) compared to the pre-calving period (Fig. 8). Afterwards, the values declined and were similar to those recorded before parturition.

The percentage of PBMC expressing TLR-4 declined in the postpartum period, and the lowest value was recorded at day 21 after calving (Fig. 9). Mean values recorded after 7,

Fig. 4 Changes of plasma glucose concentrations in peri-parturient dairy cows. Plasma was separated from jugular blood of 35 Holstein cows, which were bled on a weekly basis during the last 3 weeks of pregnancy and the first 35 days of lactation. Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P<0.05$)

Fig. 5 Changes of plasma nonesterified fatty acids (NEFA) concentrations in peri-parturient dairy cows. Plasma was separated from jugular blood of 35 Holstein cows, which were bled on a weekly basis during the last 3 weeks of pregnancy and the first 35 days of lactation. Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P<0.05$)

14, and 21 days of lactation were lower ($P<0.05$) compared to the pre-calving period.

Correlation analysis between parameters under investigation pointed out the following significant correlations: positive between plasma and intracellular Hsp72 ($r=0.46$, $P<0.001$), between plasma NEFA and intracellular Hsp72 ($r=0.22$, $P<0.05$), and between PBMC proliferation in response to LPS and the percentage of PBMC expressing TLR-4 ($r=0.36$, $P<0.01$), and negative between PBMC proliferation in response to LPS and plasma or intracellular Hsp72 ($r=-0.25$, $P<0.01$, and $r=-0.22$, $P<0.05$, respectively).

Discussion

The THI values recorded during the study indicated that SP and SU cows were exposed to thermohygrometric con-

Fig. 6 Changes of tumor necrosis factor-alpha (TNF- α) concentrations in peri-parturient dairy cows. Plasma was separated from jugular blood of 35 Holstein cows, which were bled on a weekly basis during the last 3 weeks of pregnancy and the first 35 days of lactation. TNF- α was determined by a cytotoxicity assay on WEHI 164 cells. Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P<0.05$)

Fig. 7 Changes of the proliferative response (optical density) of lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) in peri-parturient dairy cows. PBMC were isolated from jugular blood of 35 Holstein cows, which were bled on a weekly basis during the last 3 weeks of pregnancy and the first 35 days of lactation. LPS used was from *E. coli* 0111:B4. The proliferative response was established by an ELISA system based on the measurements of 5-bromo-2'-deoxyuridine incorporation during DNA synthesis in proliferating cells. Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P < 0.05$)

ditions corresponding to thermoneutrality ($\text{THI} < 72$) and mild hot ($72 < \text{THI} < 80$), respectively (Johnson 1987). With regard to summer THI data, even if the mean value of maximum THI was above the upper critical value of 72 (Johnson 1987), the daily values of minimum THI were always below 64, which some authors indicated as the critical minimum value, below which the consequences of heat stress in dairy cows are limited (West and Mullinix 2003). Analogously, other studies demonstrated that mild hot does not affect immunological parameters and Hsp expression or synthesis in cattle. In two previous studies on Holstein cows, we reported that moderate summer heat

Fig. 8 Percentage changes of peripheral blood mononuclear cells (PBMC) expressing CD14 receptors in peri-parturient dairy cows. PBMC were isolated from jugular blood of 35 Holstein cows, which were bled on a weekly basis during the last 3 weeks of pregnancy and the first 35 days of lactation. Percentage of PBMC expressing CD14 receptors was established by flow cytometry analyses. Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P < 0.05$)

Fig. 9 Percentage changes of peripheral blood mononuclear cells (PBMC) expressing Toll-like receptors (TLR)-4 in peri-parturient dairy cows. PBMC were isolated from jugular blood of 35 Holstein cows, which were bled on a weekly basis during the last 3 weeks of pregnancy and the first 35 days of lactation. Percentage of PBMC expressing TLR-4 was established by flow cytometry analyses. Data are reported as least square means $\pm$ standard error. Different letters indicate significant differences between time points ($P < 0.05$)

stress did not modify mitogen-driven proliferation of PBMC, immunoglobulin concentrations in colostrum, and passive immunization of newborn calves (Lacetera et al. 2002), and that incubation temperatures simulating conditions of mild hyperthermia did not affect Hsp72 gene expression in lymphocytes (Lacetera et al. 2006). Likewise, Yániz et al. (2009) reported that conditions of mild hot did not affect Hsp72 concentrations in PBMC from early or mid-pregnant cows. Finally, no effect of summer heat stress was reported in the expression of Hsp70 in lymphocytes isolated from beef cattle (Eitam et al. 2009) or dairy cows (do Amaral et al. 2010). Therefore, values of summer THI recorded during the present study are likely to justify the lack of significant differences between SP and SU cows for the parameters under investigation.

In a recent review paper, Johnson and Fleshner (2006) reported that, although a certain distinction may be made between the effects of Hsp72 based on their relative location (intracellular versus extracellular), factors that regulate concentration of intracellular or extracellular Hsp72 may not be clearly differentiated. The present study did not clarify the causes or the biological significance of the post-calving increase of both intracellular and circulating Hsp72 in dairy cows. However, literature data and changes of metabolic and immunological parameters reported herein authorize a few interpretative hypotheses, which are discussed hereunder.

Molvarec et al. (2007) reported that serum concentration of Hsp72 is lower in pregnant than in non-pregnant women, and this finding is, to some extent, in line with the lower values of Hsp72 found in the present study before calving compared to early lactation. Furthermore, previous studies indicated that Hsp72 mRNA expression in sheep myometrium (an intracellular milieu) was increased during labor

(Wu et al. 1996), and that concentration of Hsp72 in the amniotic fluid (an extracellular compartment) is increased in women with spontaneous labor at term (Chaiworapongsa et al. 2008). Whether parturition may be associated with increase of Hsp72 concentration also in extrauterine compartments, and whether it may cause a long-lasting increase of Hsp72, has not been established yet. However, the inflammatory and tissue remodelling processes in the reproductive tract extend to the early postpartum period in dairy cows (Azawi 2008), so that we cannot exclude that post-calving increase of plasma Hsp72 may be at least partially due to parturition-related phenomena.

Kristensen et al. (2004) reported that the stage of lactation affects plasma concentrations of Hsp72 in dairy cows. In particular, even if a statistically significant difference was lacking, plasma Hsp72 concentrations were higher in early- compared to mid-lactating cows. However, early lactating cows of that study were around 60 DIM and, therefore, in a more advanced stage of lactation than cows of our study. This makes a crucial difference since the first 30 days of lactation are characterized by conditions during which numerous cellular or whole-organism stressors may act as regulators of Hsp72 synthesis and release. In detail, early lactating dairy cows are likely to undergo negative energy balance, metabolic and digestive disturbances, oxidative stress, inflammatory dysfunctions, environmental stress, infections (especially endometritis and mastitis), and a variable degree of immunosuppression (Mulligan and Doherty 2008; Sordillo et al. 2009). In line with this tenet, we documented a postpartum decline of BCS and plasma glucose and an increase of plasma NEFA, which testify a condition of negative energy balance, and a significant positive correlation between plasma NEFA and intracellular Hsp72. Some studies demonstrated that caloric restriction, hypoglycaemia, or hyperlipidemia may differentially regulate Hsp expression in different body compartments. Eitam et al. (2009) reported that a prolonged low-energy diet promoted cell-specific Hsp response in bovine with a significant increase of Hsp90 but unchanged levels of Hsp70 mRNA in white blood cells, and a lower expression of Hsp70 in milk somatic cells. Interestingly, Febbraio et al. (2004) demonstrated that maintaining glucose availability during exercise attenuates the circulating Hsp72 response in healthy humans. Furthermore, a recent study demonstrated that heat shock-induced upregulation of intracellular Hsp72 alleviated insulin resistance and reduced fat accumulation in the hepatocytes (Morino et al. 2008). Therefore, we are prone to suggest that the negative energy balance and related changes of metabolic parameters may contribute to the upregulation of Hsp72 in the early lactation period, and also that Hsp72 upregulation is likely to exert an adaptive function towards excessive lipid mobilization. In this respect, in a series of previous studies (Lacetera et al.

2004, 2005), we reported that concentrations of NEFA indicating intense lipomobilization negatively affect lymphocyte functions in dairy cows, and suggested the increase of plasma NEFA as one of the possible mechanisms behind the peri-parturient immunosuppression. In vitro studies are in progress to establish whether the overload of a mixture of selected fatty acids mimicking composition of bovine plasma NEFA may regulate Hsp72 synthesis in PBMC from dairy cows.

In the present study, we documented a significant increase of plasma TNF- α in the early post-partum period, which confirms the tendency of dairy cows to develop a certain degree of systemic inflammation in the first weeks after calving. This result is in line with those from previous studies, which indicated that TNF- α responses of dairy cows to LPS increased from week3 before parturition to week2 after parturition (Røntved et al. 2005), and that TNF- α gene expression in mitogen-stimulated PBMC of peri-parturient dairy cows is higher in the first week after calving (Karcher et al. 2008). The present study also demonstrated that the proliferative response of PBMC to LPS gradually decrease after calving. Additionally, we found significant post-partum changes of CD14 and TLR-4 expression in PBMC, a positive correlation between LPS-triggered proliferation of PBMC and the percentage of PBMC expressing TLR-4, and negative correlations between LPS-triggered proliferation of PBMC and Hsp72. Decrease of PBMC proliferation in response to LPS and decline of TLR-4 in PBMC during early lactation have not been described before in dairy cows and may not thus be compared with previous findings. Conversely, results on early post-calving increase of CD14+ cells are in line with those reported by Røntved et al. (2005), who described a transient increase of CD14+ cells at the onset of lactation. Also, our results of the correlation analyses are in line with those obtained in other species (Fan and Cook 2004; Aneja et al. 2006), and would suggest that both upregulation of Hsp72 and decline of TLR-4-positive cells may play a role in the post-calving decrease of PBMC proliferation in response to LPS.

Which rationale lies behind reduced proliferation of PBMC in response to LPS? Several studies documented that the inflammatory state of early lactating dairy cows is at least partly due to Gram-negative bacterial endotoxins translocated from the reproductive or digestive tracts to the general circulation (Dirksen et al. 1985; Peter et al. 1990; Plaizier et al. 2008). Previous studies documented that repeated exposure to endotoxin is responsible for a stepwise reduced responsiveness to further LPS challenges in several animal species in terms of inflammatory cytokine release, which has been termed endotoxin tolerance (Hill et al. 1976; Bieniek et al. 1998; Kahl and Elsasser 2006; Cavaillon and Adib-Conquy 2006; Jacobsen et al. 2007;

Elsasser et al. 2008). It has been suggested that hyporesponsiveness to LPS is a mechanism for preventing excessive inflammatory conditions, and that a persisting hyporesponsiveness bears the risk of causing immune suppression and, hence, increased risk of secondary infections (Maas and Colditz 1987; Cavaillon et al. 2001; Cavaillon and Adib-Conquy 2006; Jacobsen et al. 2007; Opal 2007). To the best of our knowledge, development of endotoxin tolerance in early lactating dairy cows has not been described before. However, the well-known and above-mentioned translocation of bacterial endotoxin from the reproductive and digestive tracts to general circulation, the inflammatory state at the onset of lactation, and the high incidence of infections in the early lactation period (Mulligan and Doherty 2008; Pyörälä 2008) represent conditions, which may account for an endotoxin tolerance-like condition in early lactating dairy cows. In this scenario, we suggest that the gradual post-partum decline of the ability of PBMC to proliferate in response to LPS might be interpreted as an ancillary sign of hyporesponsiveness compatible with a state of endotoxin tolerance. Literature data referred to other species indicated that: (a) Hsp72 seem to be components of a complex network involved in the regulation and/or resolution of inflammatory events, (b) clear cause–effect relationships between Hsp72 and inflammation are not easily defined, and (c) whether Hsp72 have pro- or anti-inflammatory action depends on the micro-environments these proteins dwell in (Pockley et al. 2008). However, in the context of the post-partum inflammatory and immunosuppression state of dairy cows, upregulation of both PBMC and circulating Hsp72 observed in our model could prove to be of major importance in an adaptive view: intracellular Hsp72 might protect the organism against an excessive amplification of the inflammatory response (Fan and Cook 2004; Tang et al. 2007), whereas circulating Hsp72 might sustain fundamental circuits of the immune response by optimising antigen processing and presentation (Maridonneau-Parini et al. 1988; Asea et al. 2000; Campisi et al. 2003; Asea 2005).

Finally, even if not assessed herein, we wish to notice that previous studies documented that peri-parturient dairy cows also undergo a certain degree of oxidative stress (Bernabucci et al. 2005), and that this is probably a contributing factor to systemic inflammation, inflammation-based diseases, and immunosuppression (Sordillo et al. 2009). Furthermore, literature data referred to other species indicate that oxidative stress conditions upregulate Hsp72 (Asea 2007), and that heat-shock response protects cells from oxidative damage (Chiu et al. 2009). Therefore, we may hypothesize that the oxidative stress in cows around calving may be an additional inducer of Hsp72, and that such a response may be beneficial to maintain animal health and performances.

In conclusion, this study provided the first evidence on dairy cows that conditions of moderately hot climate do not affect concentrations of plasma Hsp72, and that both PBMC and circulating Hsp72 are subjected to changes during the peri-parturient period. Concomitant changes of metabolic and immunological parameters and literature data authorized the formulation of a few hypotheses upon causes of post-partum increase of Hsp72 and also on its potential adaptive significance. In particular, we suggest that the increase of Hsp72 after calving may be linked to metabolic, inflammatory, and immunological conditions typical of early lactating dairy cows, and that Hsp72 upregulation is likely to limit the consequences of lipid mobilization and inflammation. A series of in vitro studies are being carried out to establish possible cause-and-effect relationships between changes of intra and extracellular Hsp72, metabolic, and immune parameters in dairy cows.

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