Effects of β-hydroxybutyric acid on bovine milk leukocytes function *in vitro*

S. I. Cerone, A. S. Sansinanea and M. C. García

Departamento de Fisiopatología, Facultad de Cs. Veterinarias, UNCPBA, Tandil, Buenos Aires, Argentina

Abstract. The *in vitro* effect of different concentrations of β-hydroxybutyric acid (βHBA) on bovine milk leukocytes was examined. βHBA level similar to those found in cows with clinical ketosis induced a significant inhibitory effect on the nitroblue tetrazolium reduction as a mean of assaying the metabolic integrity of macrophages after the phorbol-mirystate- acetate and opsonized zymosan stimulation. In the same way, the H_2O_2 production after stimulation with both soluble and particulate agents decreased significantly in 33 and 26%, respectively, compared with cells incubated without ketone bodies. This result suggests a possible fault in the microbicidal oxidative activity. The macrophage phagocytosis also decreased in cells treated with different βHBA concentrations, in relation to that obtained from control cells. Neutrophils migration in agarose was determined, and the mean chemotactic response was higher when the cells were incubated with lower level or absence of ketone bodies. Considering the determined differences, we hypothesize that abnormally high levels of ketone bodies could produce a direct effect on leukocyte membranes. The induction of some modification on the receptor structure impairment the interaction ligand-receptor and this may be, in part, responsible for the higher susceptibility to local infections in mammary gland during subclinical and clinical ketosis.

Key words: Bovine — β-hydroxybutyric acid — Leukocytes — Milk

Introduction

In dairy cows after parturition, elevated blood levels of β-hydroxybutyric acid (βHBA) and other ketone bodies are observed. This is caused by negative energy balance. Many periparturient cows exhibit subclinical ketosis. Several studies have shown a link among increased levels of ketone bodies and metabolic and inflammatory diseases, such as infections, mastitis, metritis, placental retention, fatty liver syndrome, etc. (Gröhn et al. 1989; Cai et al. 1994; Paape et al. 1994).

Even when clinical signs do not appear, ketosis can affect milk production and reproduction (Andersson et al. 1991; Goff and Horst 1997). Clinical and subclinical ketosis both result in increased concentrations of ketone bodies in tissues and milk of the cows (Enjalbert et al. 2001).

It is still not clear by what mechanism hyperketonemia interferes with udder defence, but the mastitis incidence is higher and clinical symptoms are more severe from calving until 7–9 weeks of lactation, when cows come into a temporary state of physiological negative energy balance (Suriyasathaporn et al. 2000). Udder leukocytes have to ingest and kill invading pathogen in the presence of high ketone bodies. Klucinski et al. (1988) found that the phagocytosis of bacteria by milk polymorphonuclear neutrophils (PMN) and macrophages that are incubated in acetone or β-hydroxybutyrate are lower than in cell cultures without ketone bodies.

The primary phagocytic cells of the bovine mammary gland, PMN leukocytes and macrophages, have several functions in the phagocytic process, such as migration throught the extravascular space, phagocytosis and destruction of foreign agents (Kehrli and Harp 2001). When blood neutrophils undergo migration, the cytoplasmatic granules containing different types of pathogen-recognition molecules as CD14

Correspondence to: Silvia Irene Cerone, Departamento de Fisiopatología, Facultad de Cs. Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Pinto 399–7000, Tandil, Buenos Aires, Argentina
E-mail: scerone@vet.unicen.edu.ar

and CD18 (which bind to lipopolysaccharide and other proinflammatory cell wall components of bacteria), are brought to the surface of the cell where the molecules can interact with these bacterial components (Soler-Rodríguez et al. 2000).

The phagocytic destruction of bacteria is performed *via* different mechanisms as undergoing respiratory burst with generation of reactive oxygen species and oxidizing microbicidal agents, degranulation and pathogen killing. The immune function is linked to the release of oxygen radicals that simultaneously must be eliminated to prevent their toxic effects on intracellular proteins and membrane lipids (Heinecke 1999).

The aim of the present work was to evaluate the *in vitro* effect of different βHBA concentrations similar to the ones found in animals with clinical or subclinical ketosis, on functional capacities of milk macrophages and PMN, which constitute the first line of immunological defence in the mammary gland against pathogenic agents.

Materials and Methods

Isolation of milk cells

Milk samples (1 l) were collected from five Holando-Argentino non-periparturient cows without disease or sign of mammary gland inflammation and milk somatic cell count: SCC < 200,000 cell/ml. Milk was transported at 4°C in sterile bottles and centrifuged at 500 × *g* for 30 min. Pelleted somatic cells were washed 3 times with RPMI (Roswell Park Memorial Institute) 1640 medium containing 5% fetal bovine serum (FBS), penicillin (100 IU/ml) – streptomycin (100 μg/ml), resuspended in 16 ml of the same medium and layered on top of 9 ml of 9.5% sodium metrizoate, 5.6% polysaccharide gradient (sp. gr., 1.077 g/cm^3), centrifuged at 500 × *g* for 45 min at 4°C. Cells at the interface were resuspended in culture medium, identified as monocytes/macrophages for the presence of α-naphthyl acetate esterase activity (Politis et al. 1995). Cells from the bottom layer were collected and considered as neutrophils based on morphological characteristics as described McDonald and Anderson (1981). Differential cell counts on the isolates were performed on eosin-Giemsa stained smears. PMN were characterized by their multilobed or sometimes pycnotic dark-bluish stained nucleus. Macrophages typically had a large size, a vacuolated nucleus and contained fat globules in their cytoplasm. Epithelial cells were identified as large, polygonal, uniformly light-bluish stained cells. Cells were identified on at least 200 cells *per* slide. The viability was assessed by trypan blue dye exclusion (between 93–95% viable). All chemical products were from Sigma Chemical Co. (St. Louis, MO, USA) and Nycomed AS (Oslo, Norway).

Neutrophils migration

The polymorphonuclear leukocytes were collected from the bottom layer, washed twice with RPMI 1640 supplemented with 5% FBS, penicillin (100 IU/ml) – streptomycin (100 μg/ml), resuspended in RPMI 1640 at 10^7 cells/ml in the absence or presence of either 70 or 200 μmol/l of βHBA and incubated 45 min at 37°C in a humidified 5% CO_2/95% air incubator. After this, aliquots of 50 μl of neutrophils were placed into Petri dishes with 1% agarose in HBSS-HEPES, pH 6.6 with 10% FBS, with one set of three wells of 2.5 mm in diameter and separated 2.5 mm from each other. The middle well received 10 μl of neutrophils suspension, and 10 μl of the chemotactic factors opsonized zymosan (OpZ) or phorbol-12-myristate-13-acetate (PMA) or HBSS were added into the rest wells. After incubation for 3 h at 37°C and 5% CO_2 in a humidified incubator, the Petri dishes were flooded with methanol, removed the agarose and the cells were stained with May-Grünwald Giemsa. The migration activity was determined subtracting the migration distance towards the negative control from the migration distance towards the positive control. The five samples from five cows were tested in triplicate (Politis et al. 1991).

Macrophage phagocytosis

The cells were incubated in 1.9 ml of RPMI 1640 medium plus 10% FBS and 100 μl of physiological solution without βHBA (control) or with 70 μmol/l or 200 μmol/l of βHBA diluted in the same solution, plated at 10^7 cells/2 ml/well in a 12-well plastic plate with sterile glass coverslips inside and incubated at 5% CO_2 and 37°C. After 24-h incubation, the adherent cells were carefully washed twice in RPMI 1640 medium and then with PBS.

The washed cells were exposed to 100 μl aliquot of OpZ particle suspension and incubated for 45 min at 37°C in a humidified 5% CO_2/95% air incubator. After this, the cells were carefully washed twice with PBS to remove unbound zymosan particles, treated for 30 s with a mixture of tannic acid (0.5%) and 1 mol/l crystal violet in isotonic solution (2.2% glycerol in water). The coverslips were washed twice with 2.2% glycerol in water, dried, stained with May-Grünwald Giemsa and examinated under light microscopy. At least 100 macrophages were scored in triplicate culture from each cells isolated sample. Data are expressed as percentage of cells with particles displaying a purple stained core (Lombard et al. 1994).

Macrophage function test

Nitroblue tetrazolium (NBT) reduction test. The NBT is a yellow dye which, after incorporation into the phagolysosome, is biochemically transformed in a blue formazan crystal. The more metabolically active cells are the more NBT incorporated

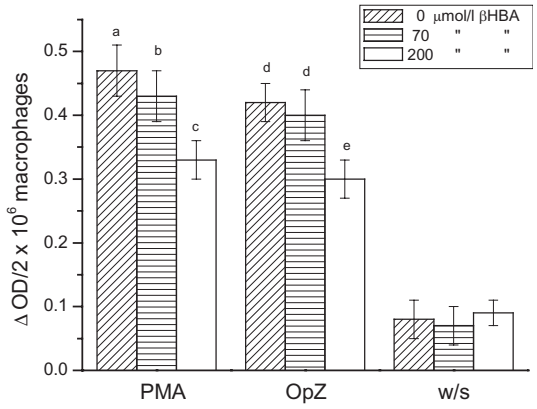

Figure 1. NBT reduction of macrophages incubated with β-hydroxybutyric acid (βHBA), expressed as Δ OD/2 $\times 10^6$ macrophages/15 min. PMA, phorbol-12-myristate-13-acetate; OpZ, opsonized zymosan; w/s, without stimulant; $^{a, b, c, d, e}$ indicate significant differences ($p < 0.05$); OD, optical density. The results are mean ± SEM from five samples, repeated three times.

(Humbert et al 1971). 200 μl NBT solution (0.11% in HBSS), 200 μl of macrophages (4×10^5) obtained from culture medium without βHBA or with 70 or 200 μmol/l of βHBA, were mixed with 40 μl OpZ or 100 μl of 10 μg/ml PMA. Duplicate tubes were made without stimulant. All tubes were incubated at 37°C for 15 min, the reaction was stopped by adding 3 ml of 0.5 N HCl. Tubes were centrifuged at 1000 × *g* for 10 min, and cells were washed twice with 3 ml of 0.5 N HCl for 5 min. The sediment was resuspended in 3 ml of dimethylformamide (DMF) and heated in a boiling water bath for 10 min, then 2 ml of 10 N KOH was added and mixed thoroughly, centrifuged and the upper DMF layer was read in OD_{710} (optical density). The results are expressed as Δ OD/2 × 10^6 macrophages/15 min (Nagahata et al. 1986). All samples were analyzed in triplicate.

Production of H_2O_2

The test was carried out three times in each isolated cell sample. The production of hydrogen peroxide (H_2O_2) was measured by the method described by Pick and Mizel (1981) with slight modifications. 200 μl of macrophages (4×10^5 cells) from culture medium as described above, were mixed with 40 μl OpZ (125 μg/ml) or 100 μl of 10 μg/ml PMA. Negative controls were made without stimulant. All tubes were incubated under an atmosphere of 5% CO_2 : 95% air at 37°C. After 1 h of incubation, a solution of phenol red and 0.2 μmol/l horseradish-peroxidase was added to the medium to quantify the H_2O_2 content. After 10 min, the reaction was stopped with 100 μl of 1 N NaOH and the amount of H_2O_2 formed was measured spectrophotometrically at 620 nm. Results are expressed as nmol $H_2O_2/10^6$ cells.

Statistical analysis

All experiments were performed in triplicate from five milk samples. Average values were expressed as the mean ± S.E.M. Different assays were analyzed for statistical significance by analysis of variance using a commercially available software version 9.1.3 (SAS Institute, Cary, NC, USA), and comparison between groups were examined with Tukey's test. Differences were considered statistically significant at $p < 0.05$.

Results

βHBA concentrations of 70 μmol/l was considered as the limit present in milk from cows with subclinical ketosis, and 200 μmol/l was the concentration determined in cows with clinical ketosis (Enjalbert et al. 2001).

The mean chemotactic responses of neutrophils, incubated with βHBA values similar to those appeared in milk from animals with clinical or subclinical ketosis, were lower than of the cells incubated in absence of ketone bodies. Therefore, migration of neutrophils was significantly ($p < 0.05$) diminished, compared to the control, as show in Table 1.

Significant difference in phagocytosis of OpZ-stimulated macrophages was observed, compared to the control cells. The percentage of phagocytosis of cells obtained from culture medium in the presence of 70 or 200 μmol/l β-hydroxybu-

Table 1. Mean of chemotactic activity of neutrophils and phagocytosis of opsonized zymosan (OpZ) by bovine mammary macrophages

βHBA (μmol/l)	0	70	200
Chemotaxis to OpZ	1.54 ± 0.27 ^{a}	1.23 ± 0.22 ^{b}	1.12 ± 0.23 ^{c}
Chemotaxis to PMA	1.41 ± 0.25 ^{a}	1.21 ± 0.19 ^{b}	1.09 ± 0.31 ^{c}
Phagocytosis to OpZ	42 ± 6 ^{a}	36 ± 5 ^{b}	34 ± 6 ^{b}

Mean ± SEM are shown from five milk samples, tested in triplicate. The chemotactic activity is expressed as the difference between the migration distance towards the control medium from the migration distance towards the chemoattractant ± SEM, and the phagocytosis is expressed as percentage of cells with particles displaying a purple stained core each 100 macrophages ± SEM. $^{a, b, c}$ indicate significant difference ($p < 0.05$); βHBA, β-hydroxybutyric acid; PMA, phorbol-12-myristate-13-acetate.

tyrate was 15 or 18% lower, respectively, compared to those obtained from cells incubated only with physiological solution without β-hydroxybutyrate. This finding implies that ketone bodies have a slight inhibitory effect on the phagocytic process, at least in the initiation of this process (Table 1).

As shown in Figure 1, the NBT reduction as a way of assaying the metabolic integrity of phagocytosing cells, in macrophages treated with 200 μmol/l of βHBA in their incubation medium was approximately 30 ± 3% (mean from five samples, performed in triplicate) lower than in macrophages incubated in physiological solution with no addition of βHBA. The decrease was observed on stimulating the cells by the use of OpZ as well as PMA. Furthermore, 70 μmol/l of βHBA in the incubation medium proved to cause falling of the NBT reduction just between 5 and 9% with regard to the controls. Results are expressed as Δ OD/2 × 10^6 macrophages/15 min.

The results in Figure 2 show that the addition of βHBA in incubation medium caused an effect in a concentration dependent manner. So, similar levels that were present in milk from cows with clinical (200 μmol/l) or subclinical (70 μmol/l) ketosis decrease significantly ($p < 0.05$) the macrophage H_2O_2 production after stimulation with soluble stimulant in 33 and 25%, respectively, compared to cells incubated only with physiological solution, without ketone bodies. After stimulation with particulate agent, the decrease was 26 and 14% lower, respectively. OpZ is a particulate stimulant and the action mechanism is different from that of a soluble agent like PMA.

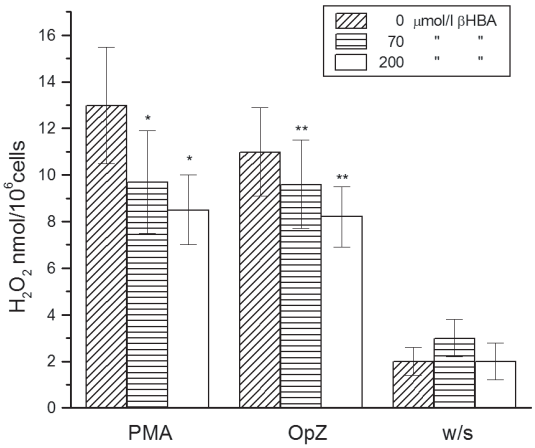

Figure 2. H_2O_2 production in macrophages incubated with β-hydroxybutyric acid (βHBA), expressed as nmol/10^6 cells. PMA, phorbol-12-myristate-13-acetate; OpZ, opsonized zymosan; w/s, without stimulant; * and ** indicate significant differences ($p < 0.05$), with respect to cells incubated without βHBA; OD, optical density. The results are mean ± SEM from five samples, repeated three times.

Discussion

Local recruitment and activity of somatic cells are the most important immune defence mechanisms against infection of the bovine mammary gland. Although a large number of milk neutrophils is critical in this control, the resident macrophages and T-lymphocytes contribute to fight successfully intramammary infection in different ways. Several interrelated defence mechanisms are deployed, which imply the release of highly toxic secretory granule components, activation of the respiratory burst, and phagocytosis. (Burton and Erskine 2003).

Blood neutrophils from lactating cows can migrate and produce significant reactive oxygen species following phagocytosis of opsonized particles. The superoxide production plays an important role in killing of invading microorganisms. Neutrophils migration from blood into the mammary subepithelium occurs as early as 2 to 4 h post infection, but peak migration into milk occurs from 8 to 12 h later as a release of cytokines peaks (Shuster et al. 1996). Delayed neutrophils recruitment into the infected mammary gland decreases the host defence mechanism against bacterial infection, and might be involved in the susceptibility of dairy cows to intramammary infection. Elevated milk level of ketone bodies in dairy cows might impair the function of neutrophils during periods of clinical and subclinical ketosis (Klucinski et al. 1988; Hoeben et al. 1997). Since a lower migration of milk neutrophils was shown in the presence of upper-normal levels of βHBA, and considering that 90% of PMN in infected glands are the PMN recruitment from blood vessels (Miller et al. 1993) would turn them unable to reach the infectious inflammatory sites.

Macrophages possess on their surfaces numerous molecules that function as specific receptors for various ligands (Detmers and Wright 1988). Several of these receptors are important for phagocytosis and cell-to-cell interactions (Kaufmann and Reddehase 1989) and are often utilized by invading microorganisms, as a mechanism of entry into host phagocytes. Compromise of macrophage Fc receptors by particle binding immunoglobulin generally leads to particle ingestion and oxidant production (Ehlenberger and Nussenzweig 1977). Likewise, the approaching of macrophage complements receptors by particle bound C3b in common mediates to particle ingestion and may promote respiratory burst and the oxidant species formation as superoxide anion and hydroxyl radicals (Shaw and Griffen 1981). Both soluble and particulate agents stimulate phagocytic cells to produce the respiratory burst. Phorbol myristate acetate is a potent soluble agent, the stimulation is independent of cell surface receptors and directly activates protein kinase C both *in vivo* and *in vitro*. Protein kinase C activity has been shown to be important for the oxidative burst of macrophages

(Johnston and Kitagawa 1985). The basal metabolism of macrophages can be significantly affected by receptor-ligand interactions, which often results in a "respiratory burst" with the metabolism of large quantities of glucose by way of the aerobic glycolysis, pentose monophosphate shunt, and an increased oxygen consumption (Adams and Hamilton 1984). Macrophages produce superoxide anion in response to stimulation by both particulate and soluble stimuli (Babior 1984). Oxygen is consumed in a reaction involving the one electron reduction of oxygen to superoxide (O_2^-). The O_2^-, thus generated, provides the microbiocidal oxidative activity because it is rapidly converted to H_2O_2 and hydroxyl radicals. As it was demonstrated in this *in vitro* assay, incubated macrophages with βHBA over normal physiological level produce less generation of H_2O_2 compared to cells without βHBA. In that way, cells challenged with OpZ reached only 65% of control value; however, when the macrophages were stimulated with PMA, the decrease was only 26% in respect to the controls. This result indicates that the possible defective phenomenon associated with generation of O_2^- is not a post-membrane pathway, assuming that OpZ triggers the oxidative metabolism through binding to cell-surface receptors (Dechatelet et al. 1982).

Although the presence of abnormally high levels of ketone bodies impairs the neutrophils migration and production of oxygen radicals during the respiratory burst, the exact mechanism is unknown; a possible explanation may be a direct effect on the leukocytes membranes and especially on membrane-bound oxidase. The presence of alcohol and carboxylic groups from βHBA give polarity to the molecule and a possibility to form hydrogen bond, which could induce some modifications on the receptor structure affecting the interaction ligand-receptor. It was recently demonstrated the involvement of ketone bodies as a factor implicated in the oxidative stress, and also they can promote lipid peroxidation in human endothelial cells *via* the generation of oxygen radicals, decrease the level of glutathione and increase peroxidation (Jain and McVie 1999). These deleterious properties could decrease the release of arachidonic acid with lower interleukin synthesis and other important products of mononuclear phagocytes as proteases, binding proteins and complement components that could impair the microbiocidal function of macrophages (Adams and Hamilton 1984).

The underlying basis for increased mastitis susceptibility may diminish the neutrophils and macrophage function, undermining a key first-line host defense against microbial infection. The knowledge of immune mechanisms involved in the mammary gland defence against invading bacteria may lead to the development of new strategies for the control of bovine mastitis.

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