**β-Hydroxybutyrate Abrogates Formation of Bovine Neutrophil Extracellular Traps and Bactericidal Activity against Mammary Pathogenic Escherichia coli***

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**Escherichia coli** is an important bacterial species isolated from bovine mastitis. The rate of neutrophil recruitment into the mammary gland and their bactericidal activity largely affect the severity and outcome of the disease. Ketosis is a common metabolic disease, and affected dairy cows are known to have increased risk for mastitis and other infectious conditions. The disease is associated with high blood and milk levels of β-hydroxybutyrate (BHBA), previously shown to negatively affect neutrophil function by unknown mechanisms. We show here that the mammary pathogenic *E. coli* strain P4 activates normal bovine neutrophils to form neutrophil extracellular traps (NETs), which are highly bactericidal against this organism. Preincubation of these neutrophils with increasing concentrations (0.1 to 8 mmol/liter) of BHBA caused a fivefold decrease of *E. coli* P4 phagocytosis, though intracellular killing was unaffected. Furthermore, BHBA caused a 10-fold decrease in the NETs formed by *E. coli* P4-activated neutrophils and a similar decrease in NET bactericidal activity against this organism. These negative effects of BHBA on bovine neutrophils might explain the increased susceptibility of ketotic cows to mastitis and other infectious conditions.

Ketosis or hyperketonemia, a condition in which blood levels of β-hydroxybutyrate (BHBA) and its metabolite acetoacetate are elevated, is common in many animal species, resulting from impaired glucose homeostasis. Important examples are diabetes in humans and other animal species, ketosis in dairy cows, pregnancy toxemia in sheep and goats, and decreased energy intake in all animal species. In both humans and farm animals, these conditions are known to be associated with increased risk for infectious diseases (14, 16, 17, 27, 28, 33, 34). Many specific defects in innate and adaptive immune functions mediated by diverse mechanisms were identified under these diverse hyperketonic conditions (29, 33, 35, 44, 45, 49). Abnormally high levels of BHBA and acetoacetate in blood and other body fluids is one of these deleterious mechanisms affecting immune functions in many animal species. BHBA was reported to affect human, bovine, and ovine neutrophil function and chemotaxis (7, 10, 20, 21, 36–38, 43, 48). Phagocytosis, microbial killing, and various antimicrobial mechanisms of neutrophils, like reactive oxygen species (ROS) production, were impaired by exposure to BHBA levels similar to those measured under hyperketonemic disease conditions.

Mastitis, an inflammatory response of the mammary tissue to invading bacteria, is a worldwide problem leading to multi-billion dollar economic losses, and *Escherichia coli* is a leading cause of acute mastitis in dairy animals. Dairy cows with elevated serum, urine, and milk levels of BHBA or acetoacetate had a significantly higher risk for mastitis (14, 31, 44). Furthermore, hyperketonemia in experimentally induced *E. coli* bovine mastitis was associated with an increased severity of disease that was attributed to neutrophil dysfunction (22). The efficacy and speed of neutrophil recruitment are the main predictors of the outcome of mammary infection. A swift response results in the rapid clearance of infection and relatively mild clinical signs (25, 32).

An important recent advancement in our understanding of neutrophil function is the discovery of extracellular neutrophil traps (NETs), which provide an additional microbial killing mechanism affecting the pathogenesis of various infectious diseases (5). NETs are extracellular structures composed of granular and nuclear neutrophil constituents that capture and kill bacteria extracellularly. Activated neutrophils release, in a process of nonapoptotic cell death, nuclear materials that mix with granular contents and are released extracellularly to form a net of DNA, nuclear proteins, and granular enzymes.

Here we show for the first time that BHBA, in concentrations corresponding to those of bovine subclinical and clinical ketosis, negatively affect the formation and function of bovine NETs against mammary pathogenic *E. coli* (MPEC) and possibly other extraintestinal pathogenic *E. coli* strains.

**MATERIALS AND METHODS**

**Isolation of blood neutrophils.** About 60 ml of heparinized blood was collected from clinically normal dairy cows by jugular venipuncture into sterile syringes and kept on ice until processing. For the isolation of neutrophils, 17.5 ml of whole blood was diluted with 22.5 ml phosphate-buffered saline (PBS) and centrifuged for 5 min at 1,500 × g at 4°C. The serum and upper third of the packed red blood cells were removed, and the remaining red blood cells were lysed for 1 min with double volumes of distilled water, followed by a fast recovery of isotonicity with 10× PBS. Neutrophils were pelleted by centrifugation at 500 × g for 3 min, resuspended in RPMI 1640 medium, and counted for viability (>90%) by trypan blue exclusion. Based on a Diff-Quik-stained cytopsin preparation, cell suspensions contained >98% polymorphonuclear leukocytes.
**Bacterial strains and culture conditions.** The *E. coli* strain P4 isolated from a case of acute bovine mastitis (3) was used in this study. The *E. coli* P4 strain is extensively used in experimental mastitis studies with dairy cows (18, 41) and mice (13, 23). *E. coli* P4 serotype O32:H37 is noncapsulated, serum resistant, motile, and highly virulent upon intramammary inoculation of dairy cows (1, 19). The plasmid pSA11 carrying lacP3 and the green fluorescent protein (GFP) gene (gfp) under the regulation of the tac promoter was introduced into *E. coli* P4, and gfp expression was achieved by isopropyl-β-D-thiogalactopyranoside or lactose (39). Bacteria were grown to log phase in Luria-Bertani (LB) broth at 37°C. Next, bacterial suspensions were diluted in sterile, nonpyrogenic PBS to the indicated concentrations and plated on LB agar to determine the CFU inoculated in every experiment.

**Neutrophil phagocytosis and bactericidal activity assays.** A suspension of 10⁶ bovine neutrophils and 10⁷ *E. coli* P4 CFU were seeded in 24-well plates in RPMI medium. Experimental conditions included medium alone, medium containing 20% heat-inactivated (56°C for 30 min) pooled normal bovine serum with or without cytochalasin D (10 µg/mL), and medium containing 100 U/mL DNase (Sigma). We have found in preliminary experiments that medium containing 20% serum or DNase completely abrogated NET formation by bovine neutrophils (data not shown). Control wells contained bacteria without neutrophils. Experiments were carried out in triplicate and repeated at least twice. Plates were incubated for 5, 10, 20, and 30 min at 37°C in a humidified CO₂ incubator in RPMI medium containing 0, 0.1, 1, 4, or 8 mmol/liter BHBA (Sigma). Neutrophils were tested for phagocytosis and bactericidal activity and NET formation as described above.

**Effect of β-hydroxybutyrate on neutrophil phagocytosis and bactericidal activity.** Bovine neutrophils were preincubated for 40 min at 37°C in a humidified CO₂ incubator in RPMI medium containing 0, 0.1, 1, 4, or 8 mmol/liter BHBA (Sigma). Neutrophils were tested for phagocytosis and bactericidal activity and NET formation as described above.

**Statistical analysis.** The percentage of phagocytosis and killing and the percentage of NET formation data are reported as the means ± standard errors of the means (SEM) of values obtained from two different experiments. Compar-
isons of the means within and between groups were tested with Student’s t tests to determine statistical significance. A P value of 0.05 or less was considered significant.

RESULTS

Phagocytosis and killing of \textit{E. coli} \textit{P4} by bovine neutrophils.

The mean (± SEM) phagocytosis activity of \textit{E. coli} \textit{P4} organisms by normal bovine neutrophils was 66% (±3.3%) at 5 min and increased to 88% (±3.4%) after 30 min. Preincubation of neutrophils with 10 μg/ml cytochalasin D completely abrogated phagocytosis (Fig. 1A). Similarly, nonopsonized \textit{E. coli} \textit{P4} organisms were not phagocytosed by normal bovine neutrophils. The mean intracellular killing of phagocytosed \textit{E. coli} \textit{P4} organisms by normal bovine neutrophils was 100% after 5 min (Fig. 1B). The mean extracellular killing of either nonopsonized \textit{E. coli} \textit{P4} organisms or by cytochalasin D-treated neutrophils increased from 37% (±3.4%) at 5 min to 50% (±8.5%) at 10 min and reached a plateau at 80% (±3.4%) killing after 20 and 30 min (Fig. 1C).

\textit{E. coli} \textit{P4}-induced NET formation by bovine neutrophils.

Normal bovine neutrophils adherent to poly-L-lysine-coated glass were infected with \textit{E. coli} \textit{P4}. NET formation was demonstrated by Sytox Orange staining of infected neutrophils, while normal, noninfected neutrophils did not form NETs at all (Fig. 2A to D). GFP-expressing \textit{E. coli} \textit{P4} cells could be seen adhering to these structures (Fig. 2C to D). The addition of 10 or 20% heat-inactivated normal bovine serum (data not shown) or of DNase to the culture medium completely eliminated NET formation (Fig. 3A) and extracellular killing of \textit{E. coli} \textit{P4} by bovine neutrophils (Fig. 3B).

\textbf{FIG. 2.} \textit{E. coli} \textit{P4} induces NET formation by bovine neutrophils. Sytox Orange DNA staining of noninfected (A) or \textit{E. coli} \textit{P4}-infected (B to D) neutrophils. NET formation (white arrows) induced by GFP-expressing \textit{E. coli} \textit{P4} cells (yellow arrows), which are seen trapped in NETs. Scale bars are 200 μm (A and B) and 50 μm (C and D).
β-Hydroxybutyrate inhibits NET formation and bactericidal effect. Preincubation of normal bovine neutrophils with increasing concentrations of BHBA inhibited NET formation and the bactericidal effect against E. coli P4 (Fig. 4). Using direct microscopic analysis, NET formation was associated with 29% of the neutrophils infected with E. coli P4. Preincubation of neutrophils with 1, 4, and 8 mmol/liter BHBA decreased NET formation to 8%, 4%, and 3.5% of neutrophils, respectively (Fig. 4).

Increasing concentrations of BHBA also decreased the NET bactericidal effect from approximately 50% to 5% of extracellular E. coli P4 organisms (Fig. 4).

**DISCUSSION**

Normal bovine neutrophils activated by various mastitis pathogens, including MPEC, were reported to produce NETs in milk (24). Also, lipopolysaccharide and interleukin 8 (IL-8), which are known neutrophil activators, were reported to induce NET formation (4, 15). Although the mechanism of NET formation by activated neutrophils is not fully elucidated, a recent report by Fuchs et al. (11) provided important insights into this process. These authors show that NET-forming neutrophils are undergoing a novel cell death process (neoapoptosis or netosis) that is distinct from apoptosis and necrosis. It should be noted that neoapoptosis is strongly dependent on ROS generation (11).

NET formation by recruited mammary neutrophils is likely to play an important role in the innate immune response against invading mammary pathogens (6, 24), and impaired NET formation or function is probably associated with increased sensitivity to mastitis pathogens. We investigated the capacity of MPEC to induce NET formation, and as expected, we found that MPEC strain P4 organisms activate bovine neutrophils to produce NETs with highly effective bactericidal activity. Importantly, we demonstrated a strong negative effect of BHBA on NET formation and bactericidal activity. Given that in hyperketonemic animals, the circulating blood neutrophils and recruited milk neutrophils are exposed to BHBA levels similar to those that repressed NET formation in vitro (40), our findings suggested that NET formation is impaired in hyperketonemic animals and that might render them more sensitive to infections.

How BHBA inhibits NET formation is not fully clear yet, and we would like to suggest three possible mechanisms to explain the negative effect of BHBA on NET formation. The first possibility is that the BHBA primary effect is inhibition of ROS formation and that it indirectly inhibits NET formation, which is dependent upon ROS formation. In agreement with this scenario, several in vitro studies reported that exposure of neutrophils to elevated levels of BHBA resulted in decreased production of ROS (20, 26).

A second explanation is that BHBA directly inhibits the process of neoapoptosis by a yet unknown mechanism. This suggestion is somewhat speculative, but it is interesting that BHBA, in concentrations similar to those inhibiting NETs, was reported to have a strong antiapoptotic and antinecrotic effect in many cell types including fibroblasts, neurons, myocytes, and glial and epithelial cells (8, 9, 30, 50). Although this effect has yet to be tested in neutrophils, it might inhibit the neoapoptotic process inducing NET formation by lipopolysaccharide-activated neutrophils.

The third possible mechanism of BHBA-mediated inhibition of NET formation is related to the BHBA tautomerase-inhibitory activity. BHBA, in concentrations similar to those inhibiting NETs, inhibits the tautomerase enzymatic activity of the macrophage migration inhibitory factor (MIF). MIF is a proinflammatory cytokine produced by neutrophils and other leukocytes (12), which play a critical role in inflammatory diseases, and the chemokine receptors CXCR2 and CXCR4 were identified as functional, noncognate, MIF receptors (2). MIF proinflammatory effects require IL8rb (which encodes CXCR2) and are dependent on its tautomerase enzymatic activity. Given the MIF CXCR2 agonistic activity and
the IL-8 stimulatory effect on NET formation, it is likely that MIF also induce NET formation. Thus, we hypothesize that by inhibiting the MIF activity, BHBA represses the capacity of the neutrophils to form NETs.

At this point, the possibility that BHBA inhibits NET formation by blocking several pathways in parallel cannot be excluded. It is possible that BHBA-mediated inhibition of ROS formation, MIF activity, and neoapoptosis all contribute to the reduction in the neutrophils' NET-forming capacity.

In line with previous studies, we showed here that bovine NETs are extremely sensitive to the destructive effect of DNase. Interestingly, various microbial pathogens adopted this strategy as a virulence mechanism, and they secrete DNase that enables them to evade killing by NETs (47). It is currently unknown if mammary pathogens also adopted this strategy. Nevertheless, given our results, an alternative microbial strategy to combat NET formation would be BHBA production and secretion. Of note, the synthesis of poly(3-hydroxybutyrate) and its degradation to BHBA are very common metabolic pathways in many bacteria including E. coli (42, 46). Therefore, the possible role of BHBA as a microbial virulence factor in mastitis and other extraintestinal infectious conditions warrants further research.

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REFERENCES


