Store-operated Ca\textsuperscript{2+} entry-sensitive glycolysis regulates neutrophil adhesion and phagocytosis in dairy cows with subclinical hypocalcemia

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ABSTRACT

Hypocalcemia in dairy cows is associated with a decrease of neutrophil adhesion and phagocytosis, an effect driven partly by changes in the expression of store-operated Ca\textsuperscript{2+} entry (SOCE)-related molecules. It is well established in nonruminants that neutrophils obtain the energy required for immune function through glycolysis. Whether glycolysis plays a role in the acquisition of energy by neutrophils during hypocalcemia in dairy cows is unknown. To address this relationship, we performed a cohort study and then a clinical trial. Neutrophils were isolated at 2 d postcalving from lactating Holstein dairy cows (average 2.83 ± 0.42 lactations, n = 6) diagnosed as clinically healthy (CON) or with plasma concentrations of Ca\textsuperscript{2+} <2.0 mmol/L as a criterion for diagnosing subclinical hypocalcemia (HYP, average 2.83 ± 0.42 lactations, n = 6). In the first experiment, neutrophils were isolated from blood of CON and HYP cows and used to analyze aspects of adhesion and phagocytosis function through quantitative reverse-transcription PCR along with confocal laser scanning microscopy, mRNA expression of the glycolysis-related gene hexokinase 2 (HKII), and components of the SOCEâ€”ORAI calcium release-activated calcium modulator 1 (ORAI1, ORAI2, ORAI3), stromal interaction molecule 1 (STIM1), and STIM2. Results showed that adhesion and phagocytosis function were reduced in HYP cows. The mRNA expression of adhesion-related syndecan-4 (SDC4), integrin β9 (ITGA9), and integrin β3 (ITGB3) and phagocytosis-related molecules complement component 1 R subcomponent (C1R), CD36, tubulinβ1 (TUBB1) were significantly decreased in the HYP group. In the second experiment, to address how glycolysis affects neutrophil adhesion and phagocytosis, neutrophils isolated from CON and HYP cows were treated with 2 μM HKII inhibitor benserazide-d3 or 1 μM fructose-bisphosphatase 1 (FBP1) inhibitor MB05032 for 1 h. Results revealed that the HKII inhibitor benserazide-d3 reduced phagocytosis and the mRNA abundance of ITGA9, and CD36 in the HYP group. The FBP1 inhibitor MB05032 increased adhesion and phagocytosis and increased mRNA abundance of HKII, ITGA9, and CD36 in the HYP group. Finally, to investigate the mechanism whereby SOCE-sensitive glycolysis affects neutrophil adhesion and phagocytosis, isolated neutrophils were treated with 1 μM SOCE activator thapsigargin or 50 μM inhibitor 2-APB for 1 h. Results showed that thapsigargin increased mRNA abundance of HKII, ITGA9, and CD36, and increased adhesion and phagocytosis in the HYP group. In contrast, 2-APB decreased mRNA abundance of HKII and both adhesion and phagocytosis of neutrophils in the CON group. Overall, the data indicated that SOCE-sensitive intracellular Ca\textsuperscript{2+} levels affect glycolysis and help regulate adhesion and phagocytosis of neutrophils during hypocalcemia in dairy cows.

Key words: subclinical hypocalcemia, neutrophils, glycolysis, adhesion, phagocytosis

INTRODUCTION

Subclinical hypocalcemia is a metabolic disorder occurring most frequently in the peripartal period that is caused by a Ca\textsuperscript{2+} imbalance (Putney, 2011). It is generally accepted that a plasma Ca\textsuperscript{2+} concentration lower than 2.00 mmol/L along with an inability to stand up define a clinical hypocalcemic state, whereas plasma Ca\textsuperscript{2+} concentrations lower than 2.00 mmol/L without clinical symptoms define subclinical hypocalcemia (Venjakob et al., 2017). Hypocalcemia disrupts reproductive performance and reduces production performance in dairy cows (Rodríguez et al., 2017). It is associated with other disorders such as metritis, displaced abomasum, retained fetal membranes, and keto-
A sensor of ER Ca\(^{2+}\) levels. Upon SOCE activation, these events trigger a downstream Ca\(^{2+}\) related signaling pathway (Putney, 2011). The Ca\(^{2+}\) receptors for regulating the concentration of Ca\(^{2+}\) in immune cells (Kimura et al., 2006). At least in nonruminants, the key mechanism involved in part by limiting the supply of glucose and Ca\(^{2+}\) for calcium metabolism during the periparturient period (Karnovsky, 1959; Borregaard and Herlin, 1982). The state of negative energy balance and derangements in calcium metabolism during the periparturient period may increase the risk of disease in dairy cows at least in part by limiting the supply of glucose and Ca\(^{2+}\) for an adequate immune response (Martinez et al., 2012).

In nonruminants, intracellular Ca\(^{2+}\) signaling is involved in cell proliferation, differentiation, migration, apoptosis, and necrosis (Martin and Bernard, 2018). A key early feature of immune cell activation is an increase in intracellular ionized calcium concentration, but the high demand for calcium by the mammary gland affects the amount of Ca\(^{2+}\) stored in the endoplasmic reticulum (ER) of immune cells (Kimura et al., 2006). At least in nonruminants, the key mechanism for regulating the concentration of Ca\(^{2+}\) in immune cells involves the store-operated Ca\(^{2+}\) entry (SOCE), a sensor of ER Ca\(^{2+}\) levels. Upon SOCE activation, Ca\(^{2+}\) accumulates in the cell membrane through the Ca\(^{2+}\) release activation channel (CRAC) (Prakriya, 2013). These events trigger a downstream Ca\(^{2+}\) related signaling pathway (Putney, 2011). The Ca\(^{2+}\) receptors in the ER are matrix interaction molecules and include stromal interaction molecule 1 (STIM1) and STIM2. When the ER Ca\(^{2+}\) is depleted, a change in conformational orientation in the ER leads to binding of STIM to CRAC and the activation of ORAI calcium release-activated calcium modulator 1 (ORAI1), ORAI2, and ORAI3. Together these processes regulate intracellular Ca\(^{2+}\) homeostasis (Park et al., 2009; Bendiks et al., 2020).

Alterations in intracellular Ca\(^{2+}\) concentrations during subclinical hypocalcemia in dairy cows alter the neutrophil recruitment cascade and its underlying molecular mechanisms (Martinez et al., 2012; Moyes et al., 2014). The proteins encoded by ORAI1 and ORAI2 regulate neutrophil activation and host defense in mice (Grimes et al., 2020), and both STIM1 and STIM2 cooperatively regulate murine neutrophil SOCE and cytokine production (Clemens et al., 2017). Although some reports have indicated that low extracellular Ca\(^{2+}\) concentrations can reduce phagocytosis in bovine neutrophils (Ducusin et al., 2003), the mechanisms controlling such an effect are unclear.

We speculate that the SOCE moiety regulates neutrophil adhesion and phagocytosis by affecting glycolysis and the pentose phosphate cycle. To address this hypothesis, the present study focused on the effects of intracellular Ca\(^{2+}\) level on changes in expression of genes associated with glycolysis along with neutrophil adhesion and phagocytosis.

**MATERIALS AND METHODS**

The Ethics Committee on the Use and Care of Animals at Heilongjiang Bayi Agricultural University (Daqing, China) approved the study protocol.

**Animals**

All cows for this study were selected from an 8,000-cow dairy farm located in Heilongjiang Province (China). They were housed in a freestall barn and had ad libitum access to a TMR (Supplemental Tables S1 and S2, https://figshare.com/articles/figure/supplemental_tables_of_JDS-22709/23731149) that was offered twice daily (0530 and 1350 h). Lactating Holstein cows (n = 30, 2 d postpartum, 2.83 ± 0.42 lactations) with normal calf delivery received a routine physical examination at 2 d postpartum by the attending veterinarian to ensure absence of other co-morbidities such as retained placenta, lameness, and subclinical mastitis. Blood samples were collected from the coccygeal vessels before feeding. One set of samples (2 mL) was immediately used to measure BHB (blood ketone meter TNN-2, Yicheng Bioelectronics Technology Co.
Isolation and Culture of Neutrophils

On d 2 postpartum, blood samples (18 mL per cow) were dispensed into 20-mL centrifuge tubes (Junnuo, Shandong, China) containing 2 mL of 3.82% sodium citrate (Solarbio, Beijing, China). After mixing evenly, the mixture was stored at low temperature and transported to the laboratory. Neutrophils were isolated according to the manufacturer’s instructions using a Bovine Peripheral Blood Neutrophil Isolation Kit (Solarbio). Briefly, blood was layered on separating solution followed by addition of PBS containing 0.02% EDTA and centrifuged at 800 × g for 10 min at room temperature. Neutrophils were collected and centrifuged at 800 × g for 10 min at room temperature. The sediment was washed with PBS and erythrocytes lysed using red cell lysing reagent followed by centrifugation for 10 min at 300 × g at room temperature. Isolated neutrophils were resuspended in 3 mL of RPMI 1640 medium after washing twice with RPMI 1640 medium (Sigma, USA).

Experimental Treatment of Neutrophils

To examine the effect of glycolysis on adhesion and phagocytosis in neutrophils, neutrophils (density of 1 × 10^6/well) from CON and HYP cows were seeded in 6-well plates with RPMI 1640 medium at 37°C with 5% CO₂ in an incubator for 1 h. Then, neutrophils were treated with vehicle (DMSO, defined as resting treatment group) or 2 μM HKII inhibitor benserazide-d3 (MedChemExpress, Monmouth Junction, NJ; Li et al., 2017), or 1 μM FBP1 inhibitor MB05032 (MedChemExpress; Quiroga et al., 2022) for 1 h, respectively.

To examine the effect of SOCE on adhesion and phagocytosis in neutrophils, neutrophils (density of 1 × 10^6/well) from CON and HYP cows were seeded in 6-well-plates with RPMI 1640 medium at 37°C with 5% CO₂ in an incubator for 1 h. Then, neutrophils were treated with vehicle (DMSO, defined as resting treatment group) or 1 μM SOCE activator thapsigargin (Beyotime Biotechnology, Shanghai, China), or 50 μM SOCE inhibitor 2-aminoethyl diphenyl borate (2-APB, Beyotime Biotechnology) for 1 h, respectively.

RNA Isolation and Quantitative Reverse-Transcription PCR

Total RNA was isolated from CON and HYP neutrophils in 6-well plates treated with or without benserazide-d3, MB05032, thapsigargin, or 2-APB using Trizol (Invitrogen Corporation, China) according to the manufacturer’s instructions, and dissolved in UltraPure Distilled Water (DNase, RNase, Free). Concentration and purity of RNA was determined with the Qubit nucleic acid protein quantitzer (Thermo Fisher, China). The mRNA (1 μg) was reverse-transcribed using the Prime Script RT Reagent Kit with gDNA Eraser kit (Takara Bio, Dalian, China) to synthesize cDNA. The mRNA abundance was detected using a SYBR fluorescent dye method (Innovagene, China). All quantitative reverse-transcription PCR reactions were run in triplicate. The reaction system contained 10 μL of SYBR Green Master, 1 μL of forward primer (10 μM) and 1 μL of reverse primer (10 μM), 2 μL of cDNA templates, and 6 μL of RNase-free distilled H₂O. The temperature program was as follows: denaturation at 95°C for 3 min, a total of 40 cycles of amplification (denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 60°C for 1 min), and finally

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<table>
<thead>
<tr>
<th>Item</th>
<th>CON (n = 6)</th>
<th>HYP (n = 6)</th>
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<tbody>
<tr>
<td>Ca (mmol/L)</td>
<td>2.30 ± 0.11</td>
<td>1.74 ± 0.10</td>
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<tr>
<td>Parity</td>
<td>2.84 ± 0.42</td>
<td>2.84 ± 0.42</td>
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<tr>
<td>Glu (mmol/L)</td>
<td>5.12 ± 1.49</td>
<td>5.80 ± 1.42</td>
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<tr>
<td>Blood ketones (mmol/L)</td>
<td>0.58 ± 0.30</td>
<td>0.47 ± 0.12</td>
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<tr>
<td>BCS</td>
<td>3.17 ± 0.49</td>
<td>3.04 ± 0.43</td>
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<tr>
<td>BW (kg)</td>
<td>618.1 ± 31.00</td>
<td>615.5 ± 66.65</td>
</tr>
<tr>
<td>Milk yield (kg/d)</td>
<td>27.43 ± 2.15</td>
<td>27.25 ± 3.01</td>
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CON = healthy controls; HYP = subclinically hypocalcemic.
Western Blot Analysis

Total protein was isolated from CON and HYP neutrophils in 6-well plates using RIPA Lysis and Extraction Buffer (Beyotime Biotechnology) including protease inhibitors. Samples were incubated on ice for 10 min after shaking, repeated 3 times, then centrifuged at 14,000 × g for 10 min at 4°C, and the supernatant was removed and used for western blotting. Protein concentration was detected with the BCA protein quantification kit, and all samples were diluted to a uniform concentration. Subsequently, equal amounts of protein (30 μg/lane) were separated on 10% SDS-PAGE and electro-transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Corp., USA; Nallagangula et al., 2017), and blocked with 5% nonfat dry milk for 45 min at room temperature. Anti-polyclonal rabbit or mouse antibody (mouse anti-ORAI1, 35 kDa, 1:1,000, ab111960, Abcam; rabbit anti-ORAI2, 29 kDa, 1:1,000, ab155216, Abcam; rabbit anti-ORAI3, 31 kDa, 1:1,000, ab254260, Abcam; mouse anti-STIM1, 77 kDa, 1:5,000, ab57834, Abcam; rabbit anti-STIM2, 84 kDa, 1:2,500, ab181258, Abcam; rabbit anti-β-actin, 45 kDa, 1:1,000, ab179467, Abcam) was used overnight at 4°C (Zhang et al., 2019b). The antibody was recovered and washed with 1× Tris-HCl solution with Tween (TBST) 3 times for 5 min. This was followed by incubation with HRP-labeled goat anti-rabbit or mouse secondary antibody (Beyotime Biotechnology, 1:2,000) for 1 h at room temperature and washing of the membrane with 1× Tris-HCl solution with Tween for 10 min. The bands were then observed with ultrasensitive ECL developer (Hai Gene, China). Band intensity was analyzed using Image J software (Amersham Imager 600, General Electric Company) and normalized to the value of β-actin.

Adhesion Assay

After 20 μL of fetal bovine serum (per well) was used to precoat a 24-well plate with round coverslips, 1 × 10^6 neutrophils from CON and HYP cows were seeded with RPMI 1640 medium at 37°C in an incubator for 1 h. Then, neutrophils were treated with vehicle (DMSO, defined as resting treatment group), or 2 μM benserazide-d3, or 1 μM MB05032, or 1 μM thapsigargin, or 50 μM 2-APB for 1 h, respectively. Neutrophils were then fixed with 2% paraformaldehyde for 15 min at room temperature and stained with Hoechst 33342 (Beyotime Biotechnology) after washing with PBS (Zhang et al., 2019a). Images were obtained with a confocal laser scanning microscope (Leica TCS SP8; Leica, Wetzlar, Germany) and analyzed with the instrument’s software.

Phagocytosis Assay

A total of 1 × 10^5 neutrophils from CON and HYP cows were seeded with RPMI 1640 medium at 37°C in an incubator for 1 h. Then, neutrophils were treated with vehicle (DMSO, defined as resting treatment group) or 2 μM benserazide-d3, or 1 μM MB05032, or 1 μM thapsigargin, or 50 μM 2-APB for 1 h, respectively. Then, cells were cultured with fluorescein isothiocyanate (FITC)-labeled *Staphylococcus aureus* at 37°C for 1 h and then placed on ice for 10 min to terminate phagocytosis (Choudhury et al., 2019; Zhang et al., 2019a). After washing twice in PBS, the samples were fixed with 2% paraformaldehyde at room temperature for 15 min (Boero et al., 2021), then the membrane was stained with DIL (Yisheng Biotechnology, China) reagent and the nucleus with Hoechst 33342. Images were obtained with a confocal laser scanning microscope (Leica TCS SP8; Leica, Wetzlar, Germany) and analyzed with the instrument’s software.

Flow Cytometry Detection

To measure cytoplasmic Ca^{2+} concentrations, neutrophils from CON (n = 6) and HYP (n = 6) cows in 6-well plates treated with or without thapsigargin or 2-APB were washed with Tyrode buffer (pH 7.4). Then, cells were incubated with 3 μM Fluo-3AM (Beyotime Biotechnology, Beijing, China) at 37°C for 30 min (Papaianniou et al., 2016). Relative fluorescence values were detected with a Beckman CytoFLEX FCM (Beckman Coulter, USA) and analyzed with the FlowJo Software, version 10 (Becton Dickinson; 2019).
Statistical Analysis

Information on the CON and HYP cows (Table 1) was summarized using descriptive statistics. Neutrophil data are presented as the means ± standard error of the means of triplicates from each sample from each cow for each assay, with n representing the number of independent experiments. The data were analyzed with IBM SPSS Statistics 26 (IBM Corp.) and reported using GraphPad Prism 7 software (GraphPad Software Inc.). To compare groups, all data were evaluated for normality with the Shapiro-Wilk test and homogeneity of variances with the Levene test, and results indicated that they were normally distributed (P > 0.05) and had homogeneous variances (P > 0.05). Subsequently, data of adhesion, phagocytosis, mRNA and protein abundance, and cytosolic calcium from HYP and CON cows were analyzed via unpaired Student’s t-tests. Neutrophil treatment comparisons were assessed by 2-way ANOVA when there were 2 factors (hypocalcemia and benserazide-d3; hypocalcemia and MB05032; hypocalcemia and thapsigargin; hypocalcemia and 2APB) and multiplicity for each variable was adjusted with the Tukey post hoc test. Statistical significance was declared if P ≤ 0.05 and a tendency was considered if 0.05 < P < 0.1.

RESULTS

Adhesion and Phagocytosis of Neutrophils in HYP Dairy Cows

Compared with CON, the mRNA abundance of adhesion-related molecules SDC4 (P = 0.006), ITGA9 (P = 0.002), and ITGB3 (P = 0.005) was lower in HYP dairy cows (Figure 1A). Immunofluorescence images revealed that the number of adherent neutrophils in the HYP group was also lower than the CON group (P = 0.042, Figure 1C-D).

Compared with CON, the mRNA abundance of the phagocytosis-related molecules C1R (P = 0.031), CD36 (P = 0.024), and TUBB1 (P = 0.038) was lower in HYP dairy cows (Figure 1B). Fluorescence intensity of phagocytosis in neutrophils of HYP dairy cows was lower than the CON group (P = 0.007, Figure 1E-F).

Effects of Glycolysis on Neutrophil Adhesion and Phagocytosis

As illustrated in Figure 2A-B, mRNA abundance of key glycolysis enzyme HKII in HYP cows was significantly lower than the CON group (P = 0.050, Figure 2A). Furthermore, the FBP1 inhibitor MB05032 increased HKII mRNA abundance in the HYP group (P = 0.004, Figure 2A). However, the HKII inhibitor benserazide-d3 had no significant effect on HKII mRNA in the HYP group (P = 0.216, Figure 2A).

Expression of SOCE in Neutrophils of Dairy Cows with Subclinical Hypocalcemia

The next series of experiments explored whether the SOCE participates in the signaling of neutrophil adhesion and phagocytosis in HYP cows. As illustrated in Figure 4A-B, the intracellular Ca2+ levels in neutrophils of HYP cows were significantly lower than the CON group (P = 0.006, Figure 4A-B). The mRNA abundance and protein expression of ORAI1, ORAI2, ORAI3, STIM1, and STIM2 in neutrophils of HYP cows were lower than the CON group (P < 0.05, Figure 4C-I).

Effects of SOCE on Glycolysis Regulates Adhesion and Phagocytosis in Neutrophils

As illustrated in Figure 5A-B, thapsigargin increased intracellular Ca2+ levels (P = 0.001) and SOCE moiety ORAI1 (P = 0.001), ORAI2 (P = 0.001), ORAI3 (P = 0.012), STIM1 (P = 0.011), and STIM2 (P = 0.012) mRNA abundance in neutrophils of HYP cows (Figure 5C-G). In contrast, 2-APB decreased intracellular Ca2+ levels (P = 0.000, Figure 6A-B) and SOCE moiety ORAI1 (P = 0.003), ORAI3 (P = 0.022), and STIM1 (P = 0.001) mRNA abundance in neutrophils of HYP cows (Figure 6C-G).

As illustrated in Figure 7A-E, thapsigargin increased the mRNA abundance of HKII (P = 0.001), ITGA9 (P = 0.023), and CD36 (P = 0.001) in neutrophils of HYP cows. Both adhesion (P = 0.016, Figure 7C,D) and phagocytosis (P = 0.011, Figure 8A,B) were enhanced by thapsigargin in neutrophils of HYP cows. Incubation with 2-APB decreased the mRNA abundance of HKII (P = 0.002) in neutrophils of HYP cows. Both adhesion (P = 0.001, Figure 7C,D) and phagocytosis (P = 0.002, Figure 8C,D) were decreased by 2-APB in neutrophils of HYP cows.

DISCUSSION

Subclinical hypocalcemia in dairy cows caused by an imbalance in Ca2+ metabolism during the transition

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Figure 1. Adhesion and phagocytosis of neutrophils in control (CON) and subclinically hypocalcemic (HYP) dairy cows. (A) Relative mRNA abundance of adhesion-related molecules (means ± SEM; n = 6) in the neutrophils of CON (white bar) and HYP (black bar) cows (SDC4, P = 0.006; ITGA9, P = 0.022; ITGB3, P = 0.005). (B) Relative mRNA abundance of phagocytosis-related molecules (means ± SEM; n = 6) in the neutrophils of CON (white bar) and HYP (black bar) cows (C1R, P = 0.031; CD36, P = 0.024; TUBB1, P = 0.038). (C) Original histogram overlays of adhesion in neutrophils in CON (left image) and HYP (right image) dairy cows. Blue represents the nucleus. (D) Quantification of adhesion in neutrophils of CON and HYP dairy cows (n = 6; P = 0.042) via fluorescence intensity. (E) Original histogram overlays of phagocytosis in neutrophils in CON (top image) and HYP (bottom image) dairy cows. Blue represents nucleus (left images), green represents Staphylococcus aureus (second images), orange represents the cell membrane (third images), merge (right images). (F) Fluorescence intensity of phagocytosis in neutrophils of CON and HYP dairy cows (n = 6; P = 0.007). *P ≤ 0.05 and **P ≤ 0.01 denote significant differences (Tukey post hoc test and Student’s t-test for analysis).
period is commonly associated with inflammation-related disorders such as retained placenta, mastitis, and metritis (Cheng et al., 2013). Subclinical hypocalcemia reduced neutrophil activity and neutrophil extracellular trap formation through decreased intracellular Ca\(^{2+}\) (Zhang et al., 2019a, 2022). In the present study, transcription markers of adhesion and phagocytosis along with the functional assays in neutrophils isolated from dairy cows with subclinical hypocalcemia were both decreased. Furthermore, our results revealed a novel mechanism whereby changing the rate of glycolysis through SOCE-regulated intracellular Ca\(^{2+}\) entry could affect neutrophil adhesion and phagocytosis.

During an inflammatory response, neutrophils accumulate on inflammatory centers through adhesion (Bouti et al., 2021) to activate a series of bactericidal mechanisms (Iqbal et al., 2022). In the process of adhesion and induced by the physical interaction between L-selectin on neutrophils, ITGA9 (one of the integrin family members required for adhesion; (van de Vijver et
Figure 3. Effect of glycolysis on neutrophil phagocytosis. (A) Original histogram overlays of phagocytosis in neutrophils of control (CON; upper images) and subclinically hypocalemic (HYP; second images) dairy cows incubated without or with benzerazide-d3 (CON + benzerazide-d3 [third images], HYP + benzerazide-d3 [lower images]). Blue represents nucleus (left images), green represents *Staphylococcus aureus* (second images), orange represents the cell membrane (third images), merge (right images). (B) Quantification of phagocytosis in neutrophils of CON (white bar) and HYP (black bar) dairy cows incubated without (*P* = 0.001) or with benzerazide-d3 are quantified (*P* = 0.045) via fluorescence intensity (means ± SEM; n = 6). (C) Original histogram overlays of phagocytosis in neutrophils of CON (upper images) and HYP (second images) dairy cows incubated without or with MB05032 (CON + MB05032 [third images], HYP + MB05032 [lower images]). Blue represents the nucleus, green represents *Staphylococcus aureus*, orange represents the cell membrane, merge (right images). (D) Quantification of phagocytosis in neutrophils of CON and HYP dairy cows incubated without (*P* = 0.006) or with MB05032 (*P* = 0.008) via fluorescence intensity (means ± SEM; n = 6). *P* ≤ 0.05 and **P** ≤ 0.01 denote significant differences (Tukey post hoc test for analysis).
Figure 4. Expression of the store-operated Ca\textsuperscript{2+} entry (SOCE) moiety in neutrophils of dairy cows with subclinical hypocalcemia. (A) Original histogram overlays of intracellular Ca\textsuperscript{2+} level neutrophils in control (CON; left image) and subclinically hypocalcemic (HYP; right image) dairy cows. FL1:H = fluorescence high, indicating Ca\textsuperscript{2+} fluorescence. (B) Fluorescence intensity (means ± SEM; n = 6) of intracellular Ca\textsuperscript{2+} level in neutrophils of CON (white bar) and HYP (black bar) dairy cows (\(P = 0.006\)). (C) mRNA abundance (means ± SEM; n = 6) of the SOCE moiety ORAI1 (\(P = 0.013\)), ORAI2 (\(P = 0.005\)), ORAI3 (\(P = 0.022\)), STIM1 (\(P = 0.021\)), and STIM2 (\(P = 0.005\)) in neutrophils of CON (white bar) and HYP (black bar) dairy cows. (D) Representative western blots of SOCE moiety ORAI1, ORAI2, ORAI3, STIM1, and STIM2 in CON (left image) and HYP (right image). (E–I) Relative abundance (means ± SEM; n = 6) of SOCE moiety ORAI1 (E; \(P = 0.011\)), ORAI2 (F; \(P = 0.041\)), ORAI3 (G; \(P = 0.035\)), STIM1 (H; \(P = 0.042\)), and STIM2 (I; \(P = 0.049\)) in CON (white bar) and HYP (black bar). *\(P \leq 0.05\) and **\(P \leq 0.01\) denote significant differences (Tukey post hoc test for analysis).
**Figure 5.** Effect of thapsigargin on mRNA expression of store-operated Ca\(^{2+}\) entry (SOCE) moiety. (A) Original histogram overlays of intracellular Ca\(^{2+}\) level in neutrophils in control (CON; upper images) and subclinically hypocalcemic (HYP; bottom images) dairy cows without or with thapsigargin. FL1:H = fluorescence high, indicating Ca\(^{2+}\) fluorescence. (B) Fluorescence intensity (means ± SEM; n = 6) of intracellular Ca\(^{2+}\) levels in neutrophils of CON (white bar) and HYP (black bar) dairy cows without (P = 0.001) or with thapsigargin are quantified (P = 0.0001). (C–G) mRNA abundance (means ± SEM; n = 6) of the SOCE moiety ORAI1 (C; P = 0.001), ORAI2 (D; P = 0.001), ORAI3 (E; P = 0.012), STIM1 (F; P = 0.011), and STIM2 (G; P = 0.012) in neutrophils of CON (white bar) and HYP (black bar) dairy cows without or with thapsigargin, respectively. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 denote significant differences (Tukey post hoc test for analysis).
Figure 6. Effect of 2-aminoethyldiphenyl borate (2-APB) on mRNA expression of the store-operated Ca\textsuperscript{2+} entry (SOCE) moiety. (A) Original histogram overlays of intracellular Ca\textsuperscript{2+} levels in neutrophils in control (CON; left images) and subclinically hypocalcemic (HYP; right images) dairy cows without or with 2-APB. FL1:H = fluorescence high, indicating Ca\textsuperscript{2+} fluorescence. (B) Quantification of intracellular Ca\textsuperscript{2+} levels in neutrophils of CON and HYP dairy cows without (\(P = 0.016\)) or with 2-APB (\(P = 0.001\)) via fluorescence intensity (means ± SEM; \(n = 6\)). (C–G) mRNA abundance (means ± SEM; \(n = 6\)) of the SOCE moiety \textit{ORAI1} (C; \(P = 0.003\)), \textit{ORAI2} (D; \(P = 0.655\)), \textit{ORAI3} (E; \(P = 0.022\)), \textit{STIM1} (F; \(P = 0.001\)), and \textit{STIM2} (G; \(P = 0.099\)) in neutrophils of CON (white bar) and HYP (black bar) dairy cows without or with 2-APB, respectively. *\(P \leq 0.05\) and **\(P \leq 0.01\) denote significant differences (Tukey post hoc test for analysis).
al., 2013; Bouti et al., 2021) is activated by Talin-1. In addition, P-selectin and E-selectin on endothelial cells also induces this interaction an contribute to enhanced adhesion of neutrophils (Yago et al., 2010). Syndecan-4 is a ubiquitously expressed transmembrane proteoglycan that co-localizes into focal adhesions along with integrins, enhancing cell attachment to extracellular matrix (Gopal et al., 2017). It has been shown that absence of SDC4 leads to smaller focal adhesions (Gopal et al., 2010, 2017). The CD36 protein, also known as scavenger receptor B2, exists in many mammalian cells and binds to toll-like receptors, which can recognize diacylglycerides in *Staphylococcus aureus* cell walls and promote bacterial phagocytosis and cytokine phagocytosis (Hoebe et al., 2005; Stuart et al., 2005). Thus, the observed decrease in both adhesion and phagocytosis of neutrophils in the HYP group underscored the reduction in immune function in those cows.

Glucose metabolism within neutrophils generates ATP via glycolysis (Sadiku et al., 2021). The protein encoded by *HKII* is an enzyme that catalyzes hexose phosphorylation. It is the first and rate-limiting enzyme in glycolysis and generates glucose-6-phosphate (G6P), an intermediate that participates in the pentose phosphate pathway and also glycolysis. The enzyme G6P dehydrogenase (G6PD) converts G6P into 6-phosphogluconate to generate NADPH, which is the main intracellular reducing agent that helps prevent oxidant damage.

**Figure 7.** Effect of store-operated Ca<sup>2+</sup> entry (SOCE)-dependent glycolysis on adhesion in neutrophils. (A–B, E) mRNA abundance (means ± SEM; n = 6) of *HKII* (A; resting *P* = 0.001, thapsigargin *P* = 0.001, 2-aminoethyldiphenyl borate [2-APB] *P* = 0.002), *ITGA9* (B; resting *P* = 0.001, thapsigargin *P* = 0.023, 2-APB *P* = 0.486), *CD36* (E; resting *P* = 0.007, thapsigargin *P* = 0.001, 2-APB *P* = 0.097) in control (CON; white bar) and subclinically hypocalcemic (HYP; black bar) dairy cows without (left image) or with thapsigargin (middle) or 2-APB (right image). (C) Original histogram overlays of adhesion in neutrophils of CON (upper image) and HYP (bottom image) dairy cows incubated without (left image) or with thapsigargin (middle image) or 2-APB (right image). Blue represents the nucleus. (D) Quantification of adhesion in neutrophils of CON and HYP dairy cows (n = 6) with resting (*P* = 0.012) and thapsigargin (*P* = 0.016) and 2-APB (*P* = 0.001) via fluorescence intensity. *P* ≤ 0.05 and **P ≤ 0.01 denote significant differences (Tukey post hoc test for analysis).
Figure 8. Effect of store-operated Ca\(^{2+}\) entry (SOCE)-dependent glycolysis on phagocytosis in neutrophils. (A) Original histogram overlays of phagocytosis in neutrophils in control (CON; upper images) and subclinically hypocalcemic (HYP; second images) dairy cows incubated without or with thapsigargin (CON + thapsigargin [third images], HYP + thapsigargin [lower images]). Blue represents the nucleus (left images), green represents Staphylococcus aureus (second images), orange represents the cell membrane (third images), merge (right images). (B) Quantification of phagocytosis in neutrophils of CON and HYP dairy cows without (\(P = 0.001\)) or with thapsigargin are quantified (\(P = 0.011\)) via fluorescence intensity (means ± SEM; \(n = 6\)). (C) Original histogram overlays of phagocytosis in neutrophils in CON (upper images) and HYP (second images) dairy cows incubated without or with 2-aminoethyl diphenyl borate (2-APB) (CON + 2-APB [third images], HYP + 2-APB [lower images]). Blue represents the nucleus (left images), green represents Staphylococcus aureus (second images), orange represents the cell membrane (third images), merge (right images). (D) Quantification of phagocytosis in neutrophils of CON and HYP dairy cows without (\(P = 0.001\)) or with 2-APB (\(P = 0.002\)) via fluorescence intensity (means ± SEM; \(n = 6\)) in neutrophils. *\(P \leq 0.05\), **\(P \leq 0.01\), and ***\(P \leq 0.001\) denote significant differences (Tukey post hoc test for analysis).
oxidative stress during glucose metabolism (Seo et al., 2021). Glucose phosphate isomerase uses G6P to form fructose-6-phosphate to continue the next steps of glycolysis (Jin et al., 2018). The regulation of FBPI, a glycolysis-related protein, can be silenced by epigenetics and play a negative role in glycolysis (Dong et al., 2013). Downregulating the expression of FBPI can enhance HIF1α expression of glucose, glucose uptake, and glycolysis pathway (Li et al., 2020). As expected, our results showed that glycolytic ability was decreased in HYP dairy cows. Further, the decrease in adhesion and phagocytosis of neutrophils in response to the glycolysis rate-limiting enzyme HKII inhibitor benserazide-d3 and the increase in adhesion and phagocytosis of neutrophils with the FBPI inhibitor MB05032 in HYP dairy cows suggested that a reduced ability of neutrophils to perform glycolysis reduces adhesion and phagocytosis capacity of neutrophils.

Previous data showed that decreased ORAI1 expression could reduce neutrophil migration, chemotaxis, adhesion, and phagocytosis in cows with subclinical hypocalcemia (Zhang et al., 2019a). Thus, the downregulation of ORAI1, ORAI2, ORAI3, STIM1, and STIM2 in the HYP group agrees with previous data. Intracellular Ca2+ can function as a feedforward signal to regulate energy metabolism including glycolysis and gluconeogenesis in nonruminant cells (Díaz-Garcia et al., 2021; Zlacká and Zeman, 2021). Thus, the observations generated with thapsigargin and 2-APB in regard to expression of the SOCE moiety components evaluated (ORAI1, ORAI2, ORAI3, STIM1, and STIM2) and intracellular Ca2+ levels indicated a similarly important function in ruminant immune cells. The fact that HKII was affected by intracellular Ca2+ concentrations in the HYP cows suggested that SOCE coordinates glycolysis in neutrophils. Furthermore, the increase in adhesion and phagocytosis of neutrophils in the HYP cows with the SOCE activator thapsigargin, and the decrease in adhesion and phagocytosis of neutrophils in response to the SOCE inhibitor 2-APB indicated that neutrophil adhesion and phagocytosis were associated with calcium levels. We speculated that Ca2+ may enter into the cells through SOCE, affecting the expression of HKII, thereby changing the rate of glycolysis and ultimately affecting the phagocytosis of neutrophils.

Although the current studies addressed SOCE-regulated glycolysis in neutrophils from cows with subclinical hypocalcemia through in vivo and in vitro studies, the complex in vivo environment during the first week postcalving including lower concentrations of insulin, thyroid hormones, glucose, and increased concentrations of free fatty acids (Overton et al., 2017) could affect calcium homeostasis or glycolysis in immune cells (Guyot et al., 2017; Zhang et al., 2019a; Venjakob et al., 2022). Thus, further research to assess ways to improve immune function of periparturient dairy cows should take into account some of these potential interactions.

In conclusion, the results from 6 clinically healthy and 6 subclinically hypocalcemic cows revealed that the level of Ca2+ in peripheral blood neutrophils mediates the glycolytic pathway to regulate the adhesion and phagocytosis capacity of the cells. These data expand the existing literature to describe the extent of immune decline during subclinical hypocalcemia in dairy cows. They also provide a scientific theoretical basis for the comprehensive evaluation of immune function characteristics of dairy cows experiencing subclinical hypocalcemia.

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