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Heat Stress Elicits Different Responses in Peripheral Blood Mononuclear Cells from Brown Swiss and Holstein Cows[1]

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ABSTRACT

This study was undertaken to assess whether peripheral blood mononuclear cells (PBMC) isolated from Brown Swiss (Br) and Holstein (Ho) cows and stimulated with concanavalin A differ in response to chronic exposure to incubation temperatures simulating conditions of hyperthermia. Five multiparous Br and 5 Ho cows were utilized as blood donors. Peripheral blood mononuclear cells were subjected for 65 h to each of 5 treatments (T). Cells were exposed to 39°C continuously (T39) and three 13-h cycles at 40 (T40), 41 (T41), 42 (T42) or 43°C (T43), respectively, which were interspersed with two 13-h cycles at 39°C. Treatment T39 was adopted to mimic normothermia; T40, T41, T42, and T43 mimicked conditions of more severe hyperthermia alternating with normothermia. Measures evaluated at the end of the incubation period were proliferative response (DNA synthesis), intracellular reactive oxygen species (ROS) concentrations, and mRNA abundance of the 72-kDa heat-shock protein (Hsp72). In Br cows, DNA synthesis began to decline when PBMC were repeatedly exposed to 41°C (–22%), whereas DNA synthesis in cells isolated from Ho cows did not begin to decline until 42°C (–40%). Furthermore, under T41 and T42, DNA synthesis from Br cows was lower than in Ho (–24 and –54%, respectively). In both breeds, increased incubation temperatures caused a reduction of intracellular ROS (from –39.6 and –69.7%). Increase in incubation temperatures enhanced Hsp72 mRNA levels only in PBMC isolated from Br cows. The Hsp72 mRNA in Br cows increased significantly under T41 and T43 compared with T39. In both breeds, DNA synthesis was positively and negatively correlated with intracellular ROS and Hsp72 mRNA abundance, respectively (r = 0.85 and r = –0.70, respectively). Results indicated that PBMC from Br cows are less tolerant to chronic heat exposure than those from Ho cows, and that the lower tolerance is associated with higher expression of Hsp72, suggesting that the same level of hyperthermia may be associated with a differential decline of immune function in the 2 breeds.

Key words: Brown Swiss, Holstein, temperature, peripheral blood mononuclear cell

INTRODUCTION

A series of in vitro studies demonstrated that exposure of bovine peripheral blood mononuclear cells (**PBMC**) to short and severe heat shock reduced responsiveness to mitogens or decreased the number of viable cells (Elvinger et al., 1991; Kamwanja et al., 1994).

Different researchers (Johnson, 1965; Correa-Calderon et al., 2004) indicated that Brown Swiss (**Br**) cows are less sensitive to hot environment exposure than Holstein (**Ho**) cows. These studies established heat sensitivity by measuring rectal temperature under hot environments and concluded that Br cows are more resistant to heat than Ho because their increase in body temperature under heat stress is less pronounced than that of Ho. To our knowledge, no in vivo or in vitro comparative studies have been conducted in breeds belonging to *Bos taurus* subspecies to establish whether different levels of hyperthermia are associated with a decline in functions of immune cells. A comparative in vitro study was performed to evaluate cellular tolerance to heat in *Bos indicus* vs. *B. taurus* breeds (Kamwanja et al., 1994); however, that study did clarify the mechanisms conferring higher tolerance to cells isolated from *B. indicus* breeds, and it was not designed to assess cell function or viability after a short-term exposure to heat-shock conditions.

Hyperthermia promotes oxidative stress in cells of laboratory animals, and that effect may be ascribed to different mechanisms, which include increased formation rate of reactive oxygen species (**ROS**; Flanagan et al., 1998). Furthermore, Pahlavani and Harris (1998) demonstrated that increased in vitro generation of oxygen free radicals due to hyperthermia was associated with inhibition of proliferation (DNA synthesis) and IL-2 gene expression in T cells from rats.

Cellular response to heat shock includes synthesis of proteins belonging to a subgroup of molecular chaperones called heat-shock proteins (**Hsp**), and classified

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into 5 families according to their molecular weight (100, 90, 70, 60, and small Hsp; Kristensen et al., 2004). The protective role of Hsp is usually confined to their chaperone function; that is, their capacity to bind denatured proteins and thus prevent their irreversible aggregation (Lindquist, 1986). In the bovine, Hsp72 is absent or expressed at a low level under nonstress conditions and is referred to as the inducible form of the Hsp70 family (Welch, 1992; Kristensen et al., 2004).

This in vitro study was undertaken to assess whether PBMC isolated from Br and Ho cows and stimulated with concanavalin A (**ConA**) differ in response to chronic exposure to temperatures simulating conditions of hyperthermia. Measures taken into consideration at the end of the incubation period were DNA synthesis (a measure of the reactivity of lymphocytes; Tizard, 1992), intracellular ROS, and Hsp72 mRNA levels.

MATERIALS AND METHODS

Cows and Samplings

Five Br and 5 Ho healthy cows originated from a commercial dairy unit, were similar for stage of lactation and parity, and were under the same management and nutritional regimen at the time of samplings. Blood collections were conducted in early spring (April 2004), during a thermoneutral period with values of the temperature humidity index constantly below 72, which is considered the upper critical value for dairy cows (Johnson, 1987). Blood samples (20 mL) were obtained 5 times over a 3-wk period by jugular venipuncture using sodium heparin (10 IU/mL) as an anticoagulant. Immediately after collection, blood samples were stored in a portable refrigerator at 4°C and transferred to the laboratory.

Laboratory Analysis

PBMC Isolation. The PBMC were isolated by density gradient centrifugation (Lacetera et al., 2002). Briefly, blood was diluted, layered over Ficoll-Paque PLUS (APB, Milano, Italy), and centrifuged. The mononuclear cell band was recovered and washed twice in PBS (Sigma, Milano, Italy). Residual red blood cells were eliminated by hypotonic shock treatment using redistilled water. The PBMC recovery and viability were determined by hemocytometer count using the trypan blue exclusion method. Viability of PBMC typically exceeded 90% both in Br and Ho cows. The PBMC were resuspended at 1×10^6 viable cells/mL in RPMI 1640 medium (Sigma) containing 25 m*M* HEPES (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, 100 U of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B/mL (Sigma). The time between blood collections and establishment of cultures was less than 6 h.

Treatments. The PBMC isolated from the 10 cows were subjected for 65 h to each of 5 treatments (**T**; Table 1). The 65-h incubation represents an optimal time to guarantee cell viability and elicit maximum proliferative response in bovine PBMC (Lacetera et al., 2005). Cells were exposed to 39°C continuously (**T39**) or three 13-h cycles at 40 (**T40**), 41 (**T41**), 42 (**T42**), or 43°C (**T43**), respectively, which were interspersed with two 13-h cycles at 39°C. Treatment T39 was adopted to mimic normothermia; T40, T41, T42, and T43 mimicked conditions of more severe hyperthermia alternated with normothermia.

PBMC Cultures for Proliferation Assay. The PBMC (100 μL) were added into triplicate wells of 96-well, flat-bottomed tissue culture plates. The mitogen ConA (Sigma) was added at final concentration of 2.5 μg/mL. Control wells contained 100 μL of PBMC suspension (1×10^6 viable cells/mL) without ConA (unstimulated). 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 subjected to the treatment protocol in an atmosphere of 95% air and 5% CO_2. After the first 48 h of incubation, 10 μ*M* of pyrimidine analogue BrdU (APB) in 10 μL of RPMI 1640 was added to each well. Following a 17-h incubation, DNA synthesis was verified by ELISA using a commercial kit (Biotrak, APB) based on the measurements of BrdU incorporation during DNA synthesis in proliferating cells. Values for DNA synthesis were expressed as the optical density recorded at 450 nm wavelength, both in unstimulated and stimulated wells, minus the optical density recorded in control wells without BrdU.

PBMC Cultures for ROS and Hsp72 Quantification. Intracellular ROS and mRNA for Hsp72 were evaluated in PBMC (1 mL of cell suspension containing 1×10^6 cells/well) cultured under the conditions described above. The PBMC were cultured in duplicate in 24-well tissue-culture plates. At the end of the incubation period, cell suspensions were transferred into centrifuge tubes and centrifuged at $1{,}000 \times g$ (g_{max}) for 15 min, the supernatants were discarded, and the dry pellets (containing whole PBMC) were stored at –80°C until analyzed.

ROS Quantification. Dry pellets of PBMC were treated with 100 μL of ice-cold lysis buffer prepared by adding 5 μL of a 10% solution of Triton X-100 and 0.5 μL of 0.2 *M* phenylmethylsulfonyl fluoride to 94.5 μL of PBS. Lysis of PBMC was performed by keeping cells on ice; cells were lysed for 15 min and were occasionally stirred. Afterwards, the cell lysate was centrifuged at