Comparison of the Insulin Reaction of Peripheral Blood T Cells between Healthy Holstein Dairy Cows and JB during the Periparturient Period

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ABSTRACT. To compare the changes in the insulin reaction of Holstein dairy cows and Japanese Black cows (JB) during the periparturient period, the insulin resistance test in vivo and lymphocytes proliferation with insulin in vitro were performed. Ten healthy Holstein dairy cows (Holstein group) and 10 healthy JB cows (JB group) used in this study were observed on days 60, 40, and 20 before calving and days 7 and 20 after calving. In insulin resistance reaction in vivo, a low insulin-stimulated glucose disposal rate and lymphocyte proliferation with insulin were observed in the Holstein group compared with the JB group during the experimental period. An analysis of the lymphocytes cultured with insulin showed that the percentage of CD4+CD45R– T cells in the Holstein group was significantly lower than that of the JB group before day 20. These findings indicate that T cells reaction to insulin in healthy periparturient Holstein cows is lower than that in Japanese Black.

KEY WORDS: Holstein dairy cow, Japanese Black, T cell.

In dairy cows, negative energy balance occurs frequently during the periparturient period, and this state is related to the onset of several diseases such as ketosis, milk fever, and abomasal displacement. Overfeeding during the dry period leads to increased levels of nonesterified fatty acids (NEFA) lipolysis in adipose tissues generating a risk of development of fatty liver [10, 13]. Decreased insulin sensitivity has been detected in hyperketotic cows with periparturient metabolic disease [8, 16]. Specifically, glucose metabolism disorder has been suggested as one important factor inducing negative energy balance in periparturient dairy cows. On the other hand, periparturient infectious diseases have been noted to occur generally in Holstein cows but not in Japanese Black cows (JB), an improved breed for beef production in Japan, and it has not been completely clarified why periparturient infectious diseases are a more common occurrence in Holstein dairy cows than in JB cattle.

Dairy cows with disorders of the metabolic system are recognized as being susceptible to infectious diseases following immuno-suppression at calving. In a previous study, the pathophysiologic mechanism of fatty liver, as a representative periparturient disease in dairy cows, was likened to non-insulin dependent diabetes mellitus in humans [11]. Humans with non-insulin dependent diabetes mellitus have a lower cellular immune response and decreased lymphocyte proliferation [6, 12]. Insulin induces potent T cell responses, which play a critical role in the immune response [3]. Although insulin is well known to be an important hormone for uptake of glucose into cells, it is not clear whether insulin affects lymphocytes in periparturient cows. In humans, the insulin sensitivity index is significantly lower in healthy African-Americans than white Americans [1]. Therefore, a marked difference in insulin sensitivity in vivo or in the activity of lymphocytes with insulin stimulation might be detected between Holstein dairy cows and JB. The aim of this study was to compare the insulin sensitivity in vivo and in vitro with the lymphocyte reaction between healthy Holstein dairy cows and healthy JB during the periparturient period.

Ten healthy Holstein dairy cows (Holstein group) in two herds and 10 healthy JB (JB group) in one herd were used in this study. The average age was 3.4 ± 0.5 years in the Holstein group and 5.4 ± 1.5 years old in the JB group. Blood samples were collected from the jugular vein. The schedule for blood collection from animals according to expected calving dates was days -60, -40, -20, and days 7, and 20 after calving. Generally, samples were collected in tubes containing dipotassium-heparin for lymphocyte culture. Plasma samples were collected with sodium fluoride for measurement of glucose (enzyme immunoassay). All samples for blood biochemical analysis were collected before the insulin resistance test.

In insulin resistance in the animals, the insulin-stimulated glucose disposal rate (GDR) was calculated using the following formula [11]:

\[ \text{GDR} \% = \frac{G0-G1}{G0} \times 100. \]

G0 is the level of blood glucose concentration before injection of insulin, and G1 is the blood glucose concentration at 30 min after intravenous injection of insulin (0.05 U/kg; Novolin R 100, Yamanouchi, Tokyo, Japan).

For lymphocyte proliferation, peripheral blood mononuclear cells (PBMCs) were seeded into 96 well microplates at
1 × 10⁷ cells/well at a final volume of 200 µl/well in RPMI 1640 medium supplemented with 2-ME, actinomycin-D and 10% fetal calf serum. Each sample was cultured with 5 µg/ml of phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO) or PHA and insulin (1 µg/ml; SIGMA). After incubation for 72 hr at 37°C, 5 mg/ml of MTT (SIGMA) was added to each well and incubation was continued for 2 hr at 37°C. After incubation, 100 µl of supernatant was removed from each well, and 100 µl of 50% N-N dimethyl sulfoxide containing 0.7 M sodium dodecyl sulfate was added. After the dark blue formazan crystals had dissolved, the plates were read on a microplate reader at a wavelength of 540 nm. The insulin-stimulated index rate of the PBMCs was calculated using the following equation:

\[
\text{Percentage} \% = \frac{\text{stimulation index of PHA contained insulin} - \text{stimulation index of PHA}}{\text{stimulation index of PHA}} \times 100.
\]

To analyze the lymphocyte population in the presence of insulin, 1 × 10⁶ PBMCs/well were cultured with 5 µg/ml of PHA containing insulin. Seventy-two hr later, cultured lymphocyte samples were washed once in phosphate-buffered saline (PBS). The lymphocytes samples were then stained with each monoclonal antibody. The used antibodies were (VMRD, Pullman, WA, U.S.A.) CACT183A (T-helper lymphocyte, CD4 antigen), BAT82A (T-cytotoxic/suppressing lymphocyte, CD8 antigen), and GC6A (non-activated leukocyte, CD45R antigen). The cells were incubated at 4°C for 60 min; then the samples were washed and the cell surface markers were visualized with goat anti-mouse IgM-fluorescein isothiocyanate and goat anti-mouse IgG-phycoerythrin isothiocyanate (Cappel, Durham, NC, U.S.A.). After 30 min incubation, the samples were washed and then analyzed. The percentage of stained cells was determined with a FACScan analyzer (Becton-Dickinson, Mountain View, CA). The insulin-stimulated index rate of the cultured lymphocyte population was calculated using the following equation:

\[
\text{Percentage} \% = \frac{\text{stimulation index of PHA contained insulin} - \text{stimulation index of PHA}}{\text{stimulation index of PHA}} \times 100.
\]

Statistics: data were analyzed with Student’s t-test and differences were judged to be significant at p<0.05. Values are expressed as means ± standard error.

A longer lasting significantly lower GDR was observed in the Holstein group compared with the JB group during the course of the study, except on day –60. In the Holstein group, proliferation of lymphocytes stimulated with PHA remained relatively stable during the course of the study and it tended to be lower than in the JB group. The in vitro data also indicate an overall decline in lymphocyte proliferation stimulated with PHA containing insulin in the Holstein group compared with the JB group, and a significant difference was detected on day –40. Insulin reactions in the two groups decreased gradually after week –20 (Fig. 1).

The percentage of CD4⁺CD45R⁻ after day –60 to day 7 compared with the JB group, except day 20, and a significantly low percentage was detected on day –20. The percentage of CD4⁺CD45R⁻ in the Holstein group increased after day –60 to day –40 and after day 7 to day 20, but no significant differences were detected between the two groups in this experiment. The percentage of CD8⁺CD45R⁻ was stable in both groups and was not significantly different during the course of study. On the other hand, the percentage of CD8⁺CD45R⁻ in the Holstein group increased gradually after day –60 and was significantly higher than the results for the JB group on day –40 and day –20 (Fig. 2).

Insulin is an important hormone that facilitates the entry...
of glucose into muscle, adipose, and several other tissues, and also stimulates the liver to store glucose in the form of glycogen. Insulin sensitivity reduces fetal and placental glucose utilization in late pregnancy, and then the high-energy requirement of milk production induces a negative energy balance in dairy cows during early lactation [7]. The results of the present study indicate that both insulin sensitivity in vivo and in vitro with the lymphocyte reaction are markedly lower in Holstein dairy cows than in JB. This finding suggests that a reduction in the utilization of glucose, because of reduced sensitivity or responsiveness of lymphocytes to insulin, might affect immunosuppression in Holstein periparturient cows.

Patients with low insulin sensitivity show no alterations in insulin responsiveness due to glucose transport, and their basal glucose transport activity is reduced [9]. Although resting human peripheral T-lymphocytes are devoid of insulin receptors, these receptors emerge upon activation of the cells by specific antigens or mitogens in humans with low insulin sensitivity [15]. Stentz [15] suggested that insulin binding, processing and responsiveness in mitogen-activated T-cells were reflective of the donor’s glycemic status and ambient insulin levels in insulin resistant states. A previous investigation has shown that there is reduced sensitivity to insulin in overfed normal periparturient cows [10]. Depression of insulin sensitivity in peripheral tissues, including the liver, is recognized as a factor that induces fatty liver in dairy cows. Since insulin enhances energy requirements and the protein synthesis necessary for appropriate T cell functions, insulin deficiency may lead to inappropriate immunoresponses in periparturient dairy cows following a reduction of glucose transport activity in T cells.

The low percentage of CD4 +CD45R– T cells seen in the Holstein cows in this study is attributable to the decreased reaction of the CD4+ T cells with insulin on day −40, which depressed lymphocyte proliferation compared to the JB group. CD45R is expressed in non-activated T cells and when activated, T cells lose CD45R expression [2]. Both CD4+ and CD8+ T cells have insulin receptors on the T cell surface, but insulin receptor expression was detected predominantly in CD4+ cells. It has been suggested that insulin receptor bearing T cells are memory cells [14]. In addition, it has been reported that mitogen activated human T lymphocytes express cell surface insulin receptors [4]. In the present study, markedly lower levels of CD4+CD45R– or proliferation after day −40 to day −20 occurred in the Holstein dairy cows than those in the JB. These findings seem to indicate that depression of cell function is facilitated by the decreased insulin response of CD4+ T cells rather than CD8+ T cells in periparturient Holstein cows.

In this study, the mechanisms underlying insulin action in CD4+ T cells could not be clearly elucidated. Campbell et al. [5] described decreased sensitivity in mammary tissue from lactating dairy cows that was, at least in part, a result of a reduction in the number of insulin receptors. The causes of frequent occurrence of periparturient infectious diseases in mature Holstein cows might be related to insulin in immune cells, high milking ability, overfeeding before

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**Fig. 2.** Increased percentage of (a) CD4+CD45R–, (b) CD4+CD45R+, (c) CD8+CD45R–, and (d) CD8+CD45R+ in lymphocytes stimulated with PHA vs PHA containing insulin after 72 hr culture in the Holstein (○) and JB groups (●). The data shown are the mean values ± standard error. *, significant difference (P<0.05) between the two groups.
calving and/or, inherent insulin status. We believe that these findings are important for research targeting the mechanisms of immunosuppression investigating periparturient infectious diseases in dairy cows.

REFERENCES