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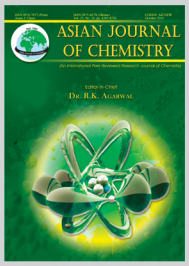

Lactogenic Hormones Regulation of Glucose Transporters Gene Expression in Lactating Bovine Mammary Epithelial Cells

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The effect of lactogenic hormones (prolactin, insulin and hydrocortisone) on expression of glucose transporters (GLUTs) gene was examined using lactating bovine mammary epithelial cells (BMEC) culture system. Compared with control (no hormone), inclusion of prolactin (10, 100 or 1000 ng/mL) and insulin (5, 50 or 500 ng/mL) did not affect abundance of GLUT1 mRNA ($P > 0.05$). Prolactin at 100 or 1000 ng/mL down-regulated, whereas insulin at all concentrations up-regulated the expression of GLUT8 mRNA ($P < 0.05$), indicating that GLUT8 may be responsive to insulin in bovine mammary epithelial cell. The abundance of GLUT1 and GLUT8 mRNA increased by hydrocortisone at 10 ng/mL, but decreased when treated at higher level (100 or 1000 ng/mL). Interaction of hydrocortisone and insulin on GLUT8 gene expression was observed ($P = 0.002$) and hydrocortisone counteracted the insulin-stimulated expression of GLUT8 mRNA. When three hormones were added together at their physiological concentrations during lactation (100 ng/mL prolactin, 5 ng/mL insulin and 100 ng/mL hydrocortisone), the expression of GLUT1 and GLUT8 mRNA was depressed ($P < 0.05$), similar to the result with 100 ng/mL of hydrocortisone. It is inferred that lactogenic hormones may not be involved in the regulation of abruptly increased expression of GLUT1 and GLUT8 mRNA in bovine mammary gland during the early lactation and hydrocortisone may play the major role in the regulation of GLUT1 and GLUT8 gene expression.

Key Words: Lactogenic hormones, Glucose transporters, Mammary epithelial cells, Bovine.

INTRODUCTION

Lactation is a complex network of physiological and metabolic changes[1,2]. The success of these processes relies on the dynamic interplay of endocrine regulation and lactogenic hormones (prolactin, insulin and hydrocortisone) play important roles. Particularly, prolactin has the commanding role in developmental regulation of the mammary gland and milk production during lactogenesis and galactopoiesis[3]; while insulin and hydrocortisone play crucial role in the transcription of the milk protein genes and are required for mammary development[4,5].

Except for hormones, the metabolic changes of nutrient also contribute to the maintenance of lactation. Glucose is an important synthetic nutrient and vital oxidative substrate for mammary metabolism. Glucose requirement increased about 4-fold in lactating dairy cows compared with their non-lactating counterparts[6], thus provision of glucose for mammary utilization is a metabolic priority. The glucose transporters (GLUTs) are responsible for the glucose supply to mammary gland, where glucose-6-phosphatase is deficient[7]. Two major glucose transporters in lactating bovine mammary gland are GLUT1 and GLUT8[8,9]. Expression of GLUTs mRNA increased about 10 to 1000 folds in bovine mammary gland from late pregnancy to early lactation[10]. Therefore, it is postulated that expression and function of GLUTs are likely to be regulated by lactogenic hormones during lactation, in order to meet the large requirement of glucose.

Regulation of GLUTs has been extensively studied, but most of the studies focus on GLUT1 in human beings and rats[11,12]. The regulations of GLUT1 and GLUT8 by lactogenic hormones in bovine mammary gland are still not clear. The aim of this study is to investigate the possible role of lactogenic hormones played in the expression of GLUT1 and GLUT8 mRNA by using lactating bovine mammary epithelial cells (BMEC) culture system.

EXPERIMENTAL

DMEM/F12 was purchased from Gibco BRL Life Technologies (Grand Island, USA). Prolactin, insulin, hydrocortisone were supplied by Sigma (St. Louis, USA); fetal calf serum was obtained from Sangon (Shanghai, China). Reagents for real-time quantitative PCR were obtained from Takara

(Tokyo, Japan). All other reagents were of the highest purity commercially available.

Isolation and culture of bovine mammary epithelial cells (BMEC): Isolation and culture of BMEC were described previously[13]. Briefly, mammary tissue of lactating Holstein dairy cow was first incubated at 37 ºC in a humidified atmosphere containing 5 % CO_2 and then BMEC migrating from the tissues were purified and collected. The cells were cultured in a modified DMEM/F12 medium (Gibco, Grand Island, USA) containing varying hormones and growth factors, to maintain the function of milk synthesis of the cells. In the experiments, the cells were digested and seeded at density of 5×10^4/mL in the 6 well culture plate.

Arrangement and treatment of experiments: When the cells grew up to about 80 % confluences, the culture media were changed to serum and hormones free medium for starvation of 24 h. Then the BMEC were treated with different concentrations of prolactin (0, 10, 100 or 1000 ng/mL), insulin (0, 5, 50 or 500 ng/mL) and hydrocortisone (0, 10, 100 or 1000 ng/mL) for 48 h, respectively. After that, the cells were harvested to isolate RNA and the RNA was reverse-transcribed to determine the abundance of GLUT1 and GLUT8 mRNA by real-time PCR. Using the same procedure, 5 ng/mL of insulin was added to each concentration of hydrocortisone (0, 10, 100 or 1000 ng/mL) to determine the interaction of insulin and hydrocortisone on GLUT8 gene expression. In addition, the cells were cultured with the combination of three lactogenic hormones at their physiological concentration during lactation (100 ng/mL prolactin, 5 ng/mL insulin and 100 ng/mL hydrocortisone) to investigate their interaction on GLUTs gene expression.

Abundance of GLUT1 and GLUT8 mRNA: Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, USA). Quality of the RNA was verified by inspection of the 18S and 28S rRNA bands after gel electrophoresis. The first strand of cDNA was performed with reverse transcription kit (Takara, Tokyo, Japan) and primers are listed in Table-1. The expression profiles of mRNA were determined by real-time quantitative PCR in a 20 -µL reaction mix containing 10 µL of SYBR Green Premix Ex Tag (7500, Applied Biosystems, Singapore).

TABLE-1
PRIMERS FOR REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Item	Gene bank accession no.	Primers
GLUT1	NM-174602	5'-GTGCTCCTGGTTCTGTTCTTCA-3' 5'- GCCAGAAGCAATCTCATCGAA-3'
GLUT8	NM-201528	5'-AGTGACTGCCCGTCCTTGCT-3' 5'-TGCTGTCCTGGCTCCTGACT-3'
β-actin	NM-173979	5'-TAGACTTCGAGCAGGAGATG-3' 5'-CCACCAGACAGCACTGTGTT-3'

Procedure of PCR reaction: 10-s of pre-denaturalization at 94 ºC, then 40 cycles of 5-s denaturation at 95 ºC and 34 s annealing and extension at 60 ºC. Optimal data were collected at the end of each extension step. All gene expression values were normalized to reference gene of β-lactin in the same sample and the $2^{-\Delta\Delta CT}$ (cycle threshold, CT) method was used to analyze the relative changes in each gene expression[14].

Statistic analysis: All the data were analyzed using SAS software system[15]. The data in effect of prolactin, insulin, hydrocortisone and combination of lactogenic hormones on GLUT1 and GLUT8 gene expression were analyzed using the ANOVA procedure. Means were determined using the least squares means statement. Duncan's multiple range tests were used for multiple comparisons. Significant differences were declared at $P < 0.05$. The data in effect of hydrocortisone and insulin on GLUT8 gene expression were analyzed using the MIXED procedure. The model included hydrocortisone, insulin and hydrocortisone × insulin interaction. Means were determined using the least squares means statement. Separation of least squares was conducted using the PDIFF option, which gives a table of *p*-values for all possible pairwise comparisons. Significant differences were declared at $P < 0.05$.

RESULTS AND DISCUSSION

Regulation of GLUT1 and GLUT8 gene expression by prolactin: The expression of GLUT1 mRNA was nearly equivalent in value at different concentrations of prolactin ($P > 0.05$. Fig. 1A), similar to the results from porcine mammary tissues[16]. Prolactin was observed to enhance the abundance of glucose transporter (GLUT2) mRNA in pancreatic β-cells[17]. The maternal insulin secretion increased during mid- to late gestation paralleling with the rise in maternal prolactin concentrations and islet prolactin receptors[18,19]. These observations suggested that prolactin may enhance glucose transport by affecting the secretion of insulin or the targeting, but not by directly influencing glucose transportation at transcriptional level in mammary gland.

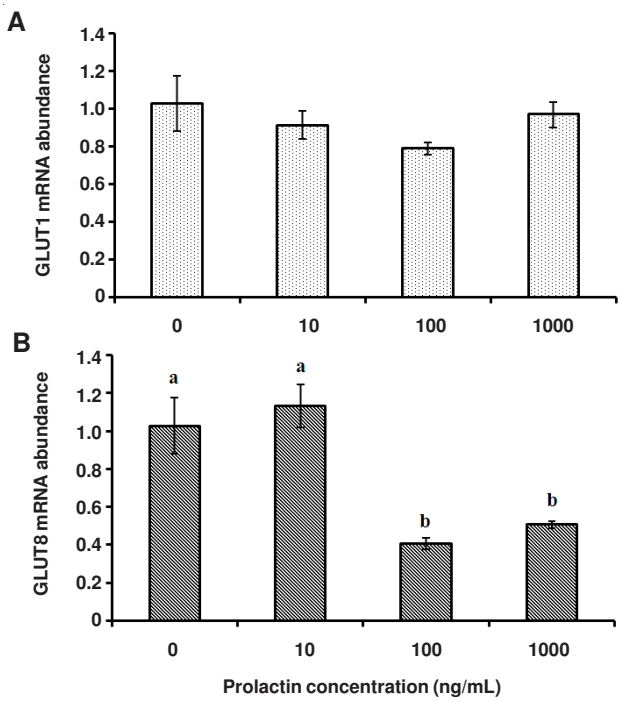

Fig. 1. Effect of different concentrations of prolactin on expression of glucose transporter 1 (GLUT1) (A) and GLUT8 (B) mRNA in bovine mammary epithelial cells. The GLUT1 and GLUT8 mRNA abundance with 0 ng/mL prolactin was assigned a value of 1.0 and the other values were expressed in relative units. Values with different superscripts (a, b) are significantly different ($P < 0.05$) and bars indicate the standard error of means (n = 4)

The abundance of GLUT8 mRNA was not affected by 10 ng/mL prolactin ($P > 0.05$), but was down-regulated by high doses of prolactin (100 or 1000 ng/mL) ($P < 0.05$, Fig. 1B). The differential expression of GLUT1 and GLUT8 mRNA regulated by prolactin may be attributed to its receptor. The prolactin receptor has at least two isoforms and only the long form is able to stimulate milk protein gene transcription[20]. Large dose of prolactin resulted in down regulation and might destroy the receptor / hormone complex[21,22], which may block the multiple signaling pathways that originate from distinct sites on the activated receptor complex[23]. There are major differences in the structures between GLUT1 and GLUT8. The primary difference is in the exoplasmic loops between transmembrane domains 1 and 2 and the carboxyl- and amino-terminal sequences are also different[9]. Because the function is closely related to the structures, it is reasonable to assume that GLUT1 and GLUT8 may respond to diverse signal transductions of prolactin receptor.

Regulation of GLUT1 and GLUT8 gene expression by insulin: Increasing concentration of insulin did not affect the abundance of GLTU1 mRNA ($P > 0.05$, Fig. 2A). The GLUT1 is widely recognized as an insulin independent glucose transporter[24,25]. Because expression of traditional insulin responsive glucose transporter (GLUT4) was not detected in the mammary gland of lactating dairy cow[25], it is deduced that mammary gland might be an insulin irresponsive organ. However, the expression of GLUT8 mRNA was increased by all concentrations of insulin compared with control ($P < 0.05$, Fig. 2B). The minimal insulin concentration that elicited a significant up-response of GLUT8 mRNA abundance was 5 ng/mL, near the plasma physiological level during lactation[26]. High concentration of insulin (50 or 500 ng/mL) did not further enhance the GLUT8 mRNA expression ($P > 0.05$, Fig. 2B), which may partly account for the increased glucose uptake in lactating bovine mammary gland at low level of insulin compared with their non-lactating counterparts[6,27]. The GLUT8 retains a dileucine motif similar to GLUT4 and might locate intracellular in bovine mammary gland[9]. Insulin stimulated the translocation of GLUT8 in blastocysts[28]. Consequently, it is inferred that the GLUT8 might be an alternative insulin responsive glucose transporter in BMEC.

Regulation of GLUT1 and GLUT8 gene expression by hydrocortisone: Hydrocortisone, a kind of glucocorticoid at 10 ng/mL significantly increased the abundance of GLUT1 and GLUT8 mRNA ($P < 0.05$, Fig. 3). Haney[29] observed that GLUT1 concentration increased about 15-fold after treated with hydrocortisone. This may due to the glucocorticoid acts as survival factors during lactation and is involved in the impairment of involution[30]. The expression of GLUT1 and GLUT8 mRNA were depressed by high concentration of hydrocortisone (100 or 1000 ng/mL) ($P < 0.05$, Fig. 3), although the plasma concentration of glucocorticoid rises significantly around parturition[31]. Excess glucocorticoid, either endogenous or exogenous, may induce abnormalities in whole-body blood glucose disposal and inhibit glucose uptake in peripheral tissues[32]. Hydrocortisone may not be involved in the up-regulation of glucose transport after parturition and the increase of hydrocortisone around parturition may be due to stress, as hydrocortisone is one of the principal hormones released in response to psychological stress[33].

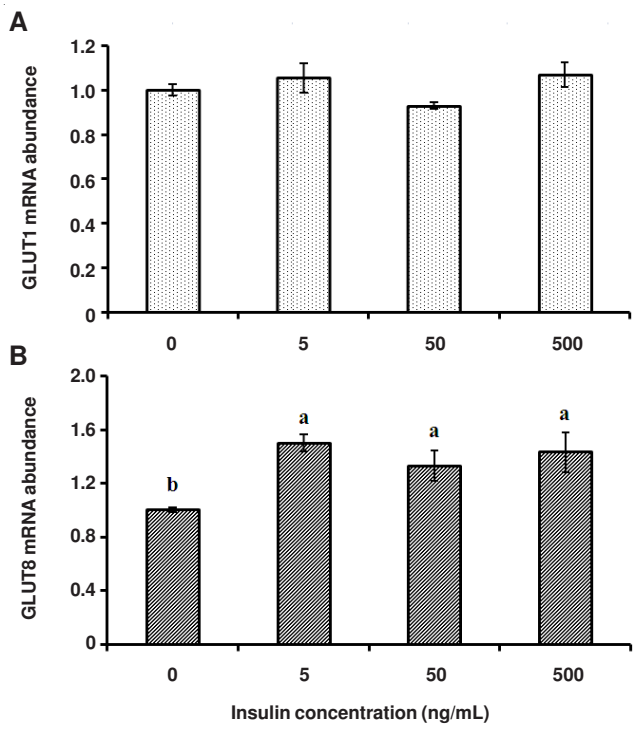

Fig. 2. Effect of different concentrations of insulin on expression of glucose transporter 1 (GLUT1) (A) and GLUT8 (B) mRNA in bovine mammary epithelial cells. The abundance of GLUT1 and GLUT8 mRNA with 0 ng/mL insulin was assigned a value of 1.0 and the other values were expressed in relative units. Values with different superscripts (a, b) are significantly different ($P < 0.05$) and bars indicate the standard error of means (n = 4)

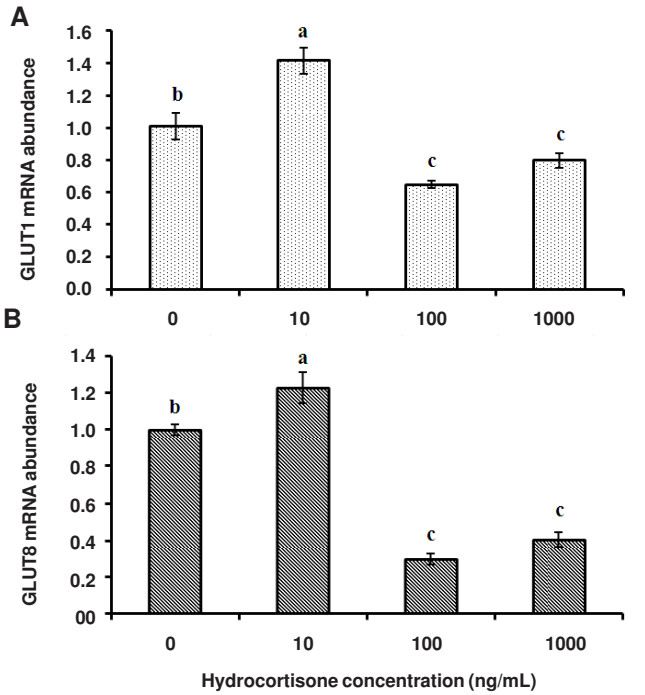

Fig. 3. Effect of different concentrations of hydrocortisone on expression of glucose transporter 1 (GLUT1) (A) and GLUT8 (B) mRNA in bovine mammary epithelial cells. The abundance of GLUT1 and GLUT8 mRNA with 0 ng/mL hydrocortisone was assigned a value of 1.0 and the other values were expressed in relative units. Values with different superscripts (a, b, c) are significantly different ($P < 0.05$) and bars indicate the standard error of means (n = 4)

Effect of lactogenic hormones on GLUT1 and GLUT8 gene expression: Effect of hydrocortisone and insulin on GLUT8 gene expression is shown in Fig. 4. Hydrocortisone affected ($P < 0.001$), while insulin did not affect the expression of GLUT8 mRNA ($P = 0.547$). Interaction of hydrocortisone and insulin on GLUT8 gene expression was observed ($P = 0.002$). Without hydrocortisone, insulin (5 ng/mL) increased the expression of GLUT8 mRNA ($P < 0.05$), while insulin with 10 ng/mL of hydrocortisone decreased the expression of GLUT8 ($P < 0.05$) compared with hydrocortisone alone. When hydrocortisone was added at higher level, insulin did not affect the expression of GLUT8 mRNA ($P > 0.05$). Glucocorticoids are reported to be negatively associated with insulin sensitivity[34] and inhibit the utilization of glucose by decreasing the translocation of glucose transporters[35]. Totally, insulin stimulated GLUT8 mRNA expression, but did not have effect on GLUT8 gene expression in the presence of hydrocortisone.

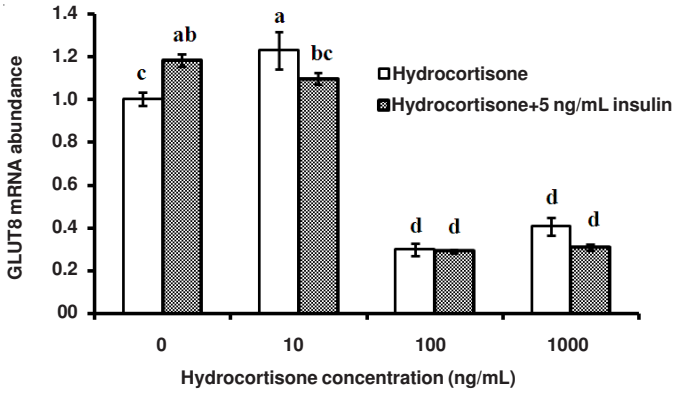

Fig. 4. Effect of insulin and hydrocortisone on expression of glucose transporter 8 (GLUT8) mRNA in bovine mammary epithelial cells. The insulin (5 ng/m) was added into different concentrations of hydrocortisone, respectively. The corresponding chart shows the mRNA abundance of GLUT8 without (open bars) and with (solid bars) insulin. The abundance of GLUT1 and GLUT8 mRNA with 0 ng/mL of hydrocortisone and insulin was assigned a value of 1.0 and the other values were expressed in relative units. Values with different superscripts (a, b, c, d) are significantly different ($P < 0.05$) and bars indicate the standard error of means ($n = 4$). Hydrocortisone, $P = 0.002$; insulin $P = 0.547$; hydrocortisone × insulin interaction, $P < 0.001$

Interactions of the three lactogenic hormones on GLUT1 and GLUT8 gene expression are shown in Fig. 5. When the three hormones (100 ng/mL prolactin, 5 ng/mL insulin and 100 ng/mL hydrocortisone) were added individually, prolactin and insulin did not affect, while hydrocortisone decreased the expression of GLUT1 mRNA ($P < 0.05$), compared with control (no hormone); the GLUT8 mRNA expression was stimulated by insulin ($P < 0.05$), but depressed by prolactin and hydrocortisone ($P < 0.05$), with the lowest value by hydrocortisone treatment. Combination of the three lactogenic hormones decreased GLUT1 and GLUT8 mRNA expression compared with control ($P < 0.05$). The GLUT1 mRNA expression with three lactogenic hormones was lower than that with hydrocortisone alone ($P < 0.05$), while the expression profile of GLUT8 was similar to that treated with hydrocortisone alone ($P > 0.05$). Cortisol was the major regulator of glucose in ruminants[36] and inhibited glucose incorporation into mammary tissue slices from lactating cows[37]. It is inferred that hydrocortisone may counteract the effects of prolactin and insulin on GLUT1 and GLUT8 gene expression and play the major role in the regulation of glucose transport gene expression among the lactogenic hormones.

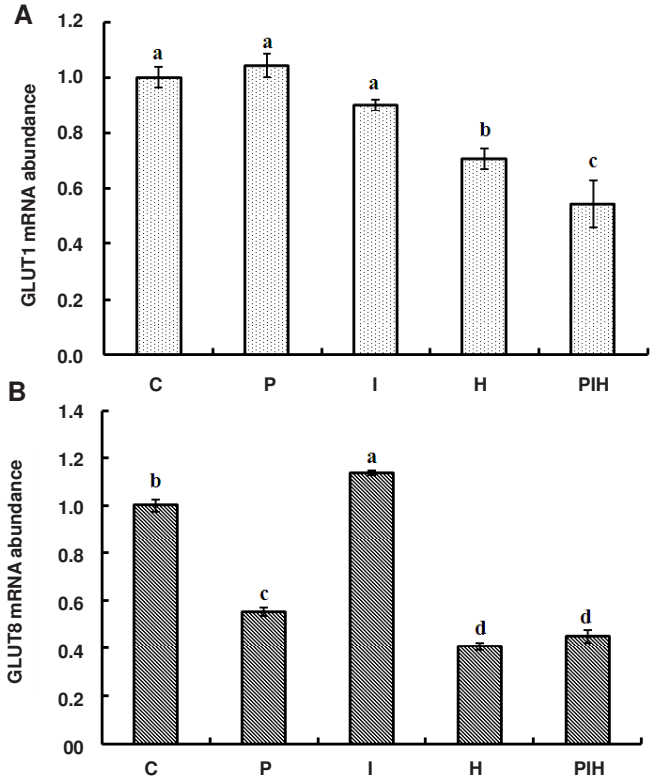

Fig. 5. Effect of lactogenic hormones on expression of glucose transporter 1 (GLUT1) (A) and GLUT8 (B) mRNA in bovine mammary epithelial cells. Cells were cultured with no hormone (C), prolactin (100 ng/mL, P), insulin (5 ng/mL, I), hydrocortisone (100 ng/mL, H) and combination of these hormones (PIH). The abundance of GLUT1 and GLUT8 mRNA with 0 ng/mL hormone was assigned a value of 1.0 and the other values were expressed in relative units. Values with different superscripts (a, b, c, d) are significantly different ($P < 0.05$) and bars indicate the standard error of means ($n = 4$)

Conclusion

Lactogenic hormones (prolactin, insulin and hydrocortisone) differentially regulated the expression of GLUT1 and GLUT8 mRNA and may play different roles in glucose transportation in bovine mammary gland. The positive interaction of prolactin, insulin and hydrocortisone on GLUT1 and GLUT8 gene expression was not observed, indicating that lactogenic hormones may not be involved in the abruptly increased expression of glucose transporter mRNA during early lactation and hydrocortisone may play the major role in the regulation of GLUT1 and GLUT8 gene expression.

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