Effects of Gut Extract Protein and Insulin on Glucose Uptake and GLUT 1 Expression in HC 11 Mouse Mammary Epithelial Cells**

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ABSTRACT : The large and rapid changes of glucose utilization in lactating mammary tissue in response to changes in nutritional state must be largely related by external signal of insulin. This also must be related with the quantity and composition of the diet *in vivo*. To characterize the mode of gut extract protein with insulin, in vitro experiment was conducted with HC11 cells. The gut extract protein has not only the same effect as insulin alone but also the synergistic effect with insulin in 2-Deoxy[3H] glucose uptake. Although the gut extract did not modulates glucose uptake via increasing the rate of translation of the GLUT1 protein, northern blot analysis indicated that the gut extract protein increased the expression of GLUT1 mRNA by a threefold and also there was a dose-dependent increase in the expression of GLUT1 mRNA. The gut extract protein is therefore shown to be capable of modulating glucose uptake by transcription level with insulin in HC 11 cells. (*Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 8 : 1210-1214*)

INTRODUCTION

The provision of glucose to the lactating mammary gland is essential for the secretion of milk. The transport of glucose into the acini cells of the lactating animal is the overall rate limiting step in the mammary-utilization of carbohydrate. The milk sugar, lactose is synthesized in the Golgi body by α-lactalbumin and galactoacyl transferase which have a Km for glucose of 1 mM which means that enzyme activity is not limiting for lactose synthesis at estimated intra-Golgi glucose concentrations of 0.1-0.3 mM. Synthesis of lactose is acutely sensitive to the nutritional state of the rat (Williamson et al., 1983), and in accordance with this, the extraction of glucose by the mammary gland, as monitored by arteriovenous glucose difference, is sensitive to starvation and refeeding (Page and Kuhn, 1986). The large and rapid changes in glucose utilization by lactating mammary tissue in response to changes in nutritional state must be largely related by external signal of insulin and this must be related to the quantity and composition of the diet. However, in lactation there is relative hypoinsulinaemia in the fed state compared with the non lactating rat (Robinson et al., 1987). Thus it has been suggested that a factor secreted by the intestine in response to feed intake might act synergistically with insulin to promote glucose utilization. A experiment have provided some evidence for an intestinal factor (Page, 1989) that enhances glucose uptake in the presence of a raised plasma insulin. The lactating mammary gland is unique in its requirement of the transport of glucose into Golgi. Glucose transporter-1 (GLUT1) is the only isoform of the glucose transporter family expressed in mammary gland. Bennett et al. (1997) reported that GLUT1 was expressed in the COMMA-D cells which is a murine mammary epithelial cell line. Thus, it is our intention to characterize the mode of a gut extract protein, which might stimulate mammary glucose uptake in the presence of insulin in HC 11 cell line.

MATERIALS AND METHODS

Cell cultures

Murine HC 11 cells were cultured in growth medium containing DMEM-F12 (Gibco BRL., USA). 10% heat-inactivated fetal bovine serum (FBS: Gibco BRL), 5 μg/ml insulin (Sigma, USA), 10 ng/ml EGF (Sigma), and 50 units/ml penicillin and 0.5 ng/ml streptomycin (Sigma). They were maintained in a humidified incubator with 95% air and 5% CO_2 at 37°C, and then, if it needed, induced to be differentiated as previously described (Doppler et al., 1989). Medium was changed every 2 days. Insulin and gut extract protein treatments were carried out as described in the figure legends. The gut extract was prepared as in Page (1989). In brief, the gastrointestinal tract was excised from fed lactating rats, cut and placed the tissue in acid/ethanol (0.1 M-HCl in 75% ethanol). The mixture was stirred fir 24 h at 5°C and centrifuged at 30,000 g for 30 min.

The supernatant was decanted, freeze-dried and stored frozen until required. The gut extract protein was used after being adjusted to pH 7 with sterile 1 M NaOH.

2-Deoxy[3H]glucose uptake

A modification of a method by Bernier et al. (1988) was used for the measurements of 2-Deoxy[3H]glucose (Deoxy-D-glucose, 2-[3H(glucose)]; 6.0 Ci/mmol; NEN Life

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Science Products, Inc.) uptake. Briefly, HC11 cells (5×10^5) were grown and/or differentiated in 24 well culture plates. After 48 h pretreatment with insulin, gut extract protein or both together in starved (DMEM without glucose, Gibco BRL, #23800) or quiescence medium, which was the same as the growth media except for no FBS and hormones, cells were washed twice with phosphate buffered saline solution (PBS). This was followed by the addition of 0.5 ml prewarmed starvation media containing 0.5 μCi 2-Deoxy[3H]glucose for 30 min at 37°C. Uptake was terminated by rapid removal of the 2-Deoxy[3H]glucose, followed by three washes in ice-cold PBS. Cells were lysed with 300 μl of 5% trichloroacetic acid; the lysates were added 3 mls of scintillant and counted in a LKB 1218 Rackbeta Liquid Scintillation counter. To measure DNA concentration, 400 μl denaturing solution was added to the well, transferred to a microfuge tube and assayed for DNA as described by Cesarone et al. (1979) by Perkin Elmer LS50 Luminescence Spectrometer. Results of 2-Deoxy[3H]glucose uptake are expressed as pmol 2-Deoxy[3H]glucose per minute per μg DNA.

Western blot analysis

After 48 h pretreatment with insulin and/or gut extract protein on 12 well culture plates, cells were washed twice with PBS and solubled in Laemmli sample buffer (Laemmli, 1970) and assayed for protein contents by BCA protein assay kit (Pierce, Rockford, IL, USA) or then subjected SDS-polyacrylamide gel electrophoresis on a gradient 12% gel and transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked for 2 h in blocking buffer (20 mM Tris-HCl, pH 7.6, 137 mM Nacl, 0.05%, Tween20, 1% polyvinylpyroiodide and 0.1% bovine serum albumin) and incubated overnight with polyclonal anti GLUT1 (Fitzgerald Industries International, Inc., Concord, MA, USA) (1:10,000 dilution in the incubating buffer) at 4°C in a incubating buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween20, 0.1% polyvinylpyroiodide and 0.1% bovine serum albumin). After a series of washes, the membrane were incubated in the incubating buffer containing rabbit anti-sheep antibodies (1:10,000 dilution in the incubating buffer) conjugated with horseradish peroxide (Upstate Biotechnology, Lake Placid, NY, USA), washed three times (30 min), immersed in enhanced chemiluminescence solution (20 mM, pH8.6 Tris, Base, 240 μg/100 ml Luminol A (Sigma), 40 μg/100 ml Coumaric acid B (Sigma) and 33.3 μl 30% H_2O_2) and exposed to autoradiography film for 1- 2 min.

RNA isolation and Northern blot analysis

After 48 h pretreatment with insulin and/or gut extract protein in 75 cm^3 flasks, total RNA was extracted by the acid/guanidium thiocyanate/phenol chloroform method (Chomozynski and Sacchi, 1987). Twenty microgram of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane. A plasmid cDNA containing mouse GLUT1 (600 bp) cloned and donated by Graeme I. Bell (Howard Hughes Medical Institute, Chicago, IL, USA) was digested with Hind III, and the insert was obtained after DEAE paper agarose gel electrophoresis. The insert of cDNA cloned was labeled using a Prime Random Primer Labelling Kit (Stratagene, La Jolla, CA, USA). The membrane was hybridized with the labeled insert of the indicated cDNA clone. The treatment differences were analyzed with a phospho image analyzer (IPR 1500, Fujifilm, Japan). The 18S and 28S ribosomal RNA bands of etidium bromide staining was then used for normalization of GLUT1 mRNA levels.

Statistical analysis

Values presented in the figures are expressed as the mean±SEM of at least three independent experiments. Differences between groups were analyzed by the Student's t-test or Duncan's multiple range test using SAS (1997; Cary, North Carolina, SAS Institute).

RESULTS AND DISCUSSION

2-Deoxy[3H]glucose uptake

Effect of cell medium : Confluent HC11 cultures were incubated in fresh growth media and changed to quiescent media with different levels of insulin for 48 h. To evaluate their response to glucose starvation, 2-Deoxy[3H]glucose uptake into cells was measured for 30 min after exposure to starved or quiescent media containing 1 μCi/ml of 2-Deoxy[3H]glucose, respectively. As shown in figure 1, The 2-Deoxy[3H]glucose uptake of cells reacted in starved media always showed more than double values ($p<0.05$ or $p<0.01$) in comparison of quiescent media. From this observation, the glucose starvation for 30 min of 2-Deoxy[3H]glucose reaction, greatly increased the glucose uptake of the cell. Transport activity in HC11 cells was probably competitively inhibited (Bennett, 1995) by 17.5 mM D-glucose in the quiescent media. So, it was decided hereafter to use the starved media for glucose uptake experiment.

Effect of cell differentiation : The HC11 cell line was isolated as a prolactin-responsive cell clone from the COMMA-1D mouse mammary epithelial cell line (Ball et al., 1988). Despite studies exploring the hormonal and substratum requirements which regulate the expression of ß-casein genes (Ball et al., 1988; Dopper et al., 1989), no studies have reported the effect of cell differentiation on glucose uptake of this cell line. As shown in figure 2, there was no difference between non-differentiated (Non-dif) and differentiated (Dif) cells at any levels of insulin (0, 0.05 and
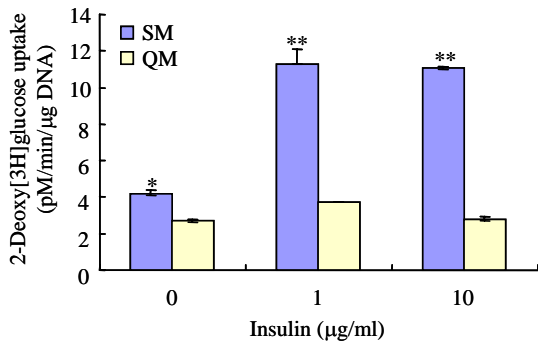
Figure 1. Effect of starved or quiescence media on glucose uptake of HC 11 cells at different levels of insulin. On the day of confluent with growth media, medium was changed to quiescence media (QM) at different levels of insulin for 24 h. 2-Deoxy[3H]glucose uptake into cells was determined 30 min after exposure to starved media (SM) or QM with no insulin addition. Each value represents the mean±SEM of triplicate determinations from three experiments. Statistical significance between cells grown in SM and QM was evaluated as described in Materials and Methods. * $p < 0.05$, ** $p < 0.01$.

0.5 µg/ml). So, it was decided hereafter to use the Non-dif cells because Non-dif cells are more time saving and economic than Dif cells for glucose uptake experiment.

Effects of gut extract protein and insulin : Figure 3 shows the gut extract protein alone has the same effect ($p < 0.05$) as insulin alone in 2-Deoxy[3H]glucose uptake. Furthermore, the gut extract protein shows the synergistic effect ($p < 0.05$) with insulin, suggesting that one or more components of the gut extract protein in the presence of insulin could be involved in the glucose uptake into the HC11 cells. In most species milk production varies according to feed intake. A ruminant will halve its rate of milk secretion within 24 h of feed withdrawal and the yields cannot be salvaged by vascular infusion of substrates (Linzell, 1967). Lactating rats respond quickly to feed withdrawal and mammary glucose uptake falls to low levels within 18 h, even though, on refeeding glucose uptake was stimulated within 15 min (Page and Kuhn, 1986). A defined gut peptide has been implicated in this response in association with insulin (Page, 1989).

Effects of gut extract protein and insulin on GLUT1 total protein level

We investigated further the effects of 48 h exposure of HC 11 cells to either gut extract protein or insulin, or both together, by measuring the total cellular content of GLUT1 protein. A western blot analysis from a representative
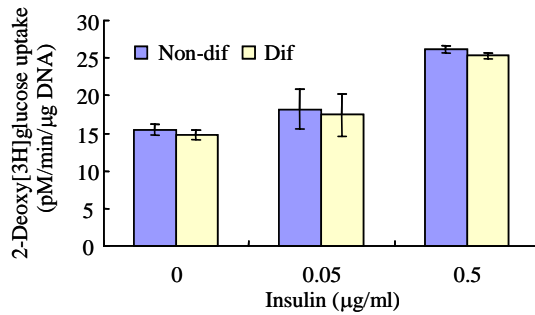

Figure 2. Effect of differentiation on glucose uptake of HC 11 cells at different levels of insulin. On the day of confluent with growth media, medium was changed to differentiation media for 4 days for differentiation cells (Dif) while non-differentiation cells (Non-dif) were kept in induction media. After that, both cells were changed to quiescence media at different levels of insulin for 24 h. 2-Deoxy[3H]glucose uptake into cells was determined 30 min after exposure to starved media. Each value represents the mean±SEM of triplicate determinations from three experiments.

| Insulin (µg/ml) | 0 | 0 | 0 | 5 | 5 | 5 |
| Gut extract protein (µg/ml) | 0 | 5 | 50 | 0 | 5 | 50 |

Figure 3. Effect of insulin and gut extract protein levels on glucose uptake of HC 11 cells. On the day of confluent with growth media, medium was changed to quiescence media (QM) at different levels of insulin and gut extract protein levels for 24 h. 2-Deoxy[3H]glucose uptake into cells was determined 30 min after exposure to starved media. Each value represents the mean± SE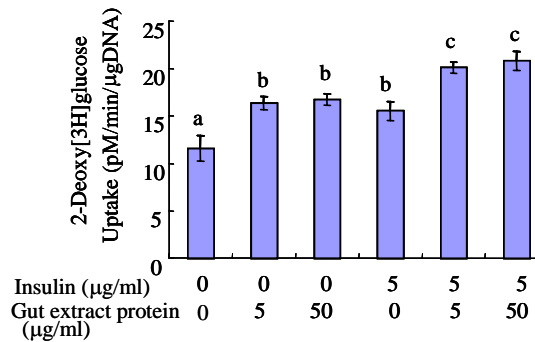M of triplicate determinations from three experiments. a,b,c means above the columns followed by different letters are significantly different ($p < 0.05$).

experiment is shown in figure 4. Inconsistent with published data of other cell lines (Wang et al., 1997), treatment with neither insulin nor gut extract protein, or both together increased GLUT1 protein content in HC 11 cells. However it could be quite due to the result of Prosser

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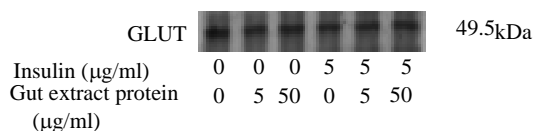
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GLUT					49.5kDa
Insulin (μg/ml)	0	0	0	5	5	5
Gut extract protein (μg/ml)	0	5	50	0	5	50

Figure 4. Effect of insulin and gut extract protein on GLUT1 protein content in HC 11 cells. HC 11 cells were cultured for 24 h in the presence or absence of various concentrations of insulin and/or gut extract protein. HC 11 cell protein preparations (15 μg) were analyzed by Western blotting for the presence of GLUT1.

(1998) who clarified that restoration of glucose transporter activity after refeeding does not appear to require the synthesis of new protein, but a change in their orientation within the plasma membrane or a redistribution within the cell. This result suggests that gut extract protein and insulin did not modulate glucose uptake via increasing the rate of translation or the protein stability of the transporter.

Effects of gut extract protein and insulin on the expression of GLUT1 mRNA

Northern blot analysis indicated a threefold increase (p<0.05) in the expression of GLUT1 mRNA by insulin in figure 5. There was also a dose-dependent increase in GLUT1 mRNA expression following a 48 h pretreatment with gut extract protein. While 5 μg/ml gut extract protein caused twofold increase in GLUT1 mRNA levels, more than fivefold increase was shown in 50 μg/ml gut extract protein treatment. On the other hand, compared with insulin alone, a 20% increase was found in insulin plus gut extract protein treatment, there was no any gut extract protein synergistic effect (p<0.05) on insulin. Moreover, 5 μg/ml gut extract protein lessened (p<0.05) the influence of insulin on GLUT1 gene expression, such that the level of GLUT1 mRNA was 38% that in insulin alone cells. This increase in expression of GLUT1 mRNA was in relatively good agreement with the increase in transport activity measured in 48 h in gut extract protein and/or insulin as in figure 3. The COMMA-D cells treated with insulin showed no significant increase in overall RNA synthesis but a specific 50% increase in GLUT1 mRNA first detected at eight hours and continuing till 24 h (Bennett, 1995). To my knowledge, however, this is the first time that the increase in GLUT1 mRNA levels was induced by insulin and/or gut extract in HC 11 cells. This finding also clarifies that a gut peptide extract stimulate mammary glucose uptake in the presence of insulin (Page, 1989) in molecular level.
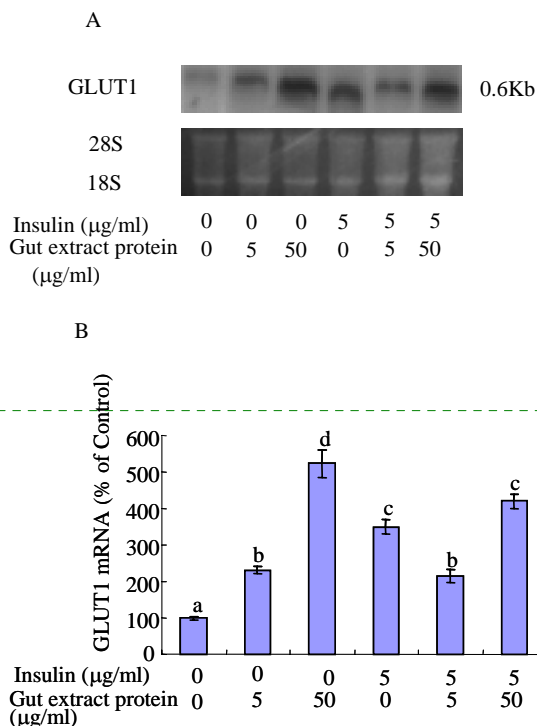
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GLUT1						0.6Kb
28S						
18S						
Insulin (μg/ml)	0	0	0	5	5	5
Gut extract protein (μg/ml)	0	5	50	0	5	50

B

[i20]: prosser

| Insulin (μg/ml) | 0 | 0 | 0 | 5 | 5 | 5 |
| Gut extract protein (μg/ml) | 0 | 5 | 50 | 0 | 5 | 50 |

Figure 5. Effect of insulin and gut extract protein on GLUT1 mRNA levels in HC 11 cells. HC11 cells cultured for 24 h in the presence or absence of various concentration of insulin and/or gut extract protein were harvested and total RNA extracted. Total RNA (20 μg) was analyzed by Northern hybridization for the presence of GLUT1 mRNA. The 18S and 28S ribosomal RNA bands of etidium bromide staining was then used for normalization of GLUT1 mRNA levels. (A) The blots represent one individual experiment, while (B) the bar graph indicates the mean±SEM of three independent Northern blot analyses. a,b,c,d means above the columns followed by different letters are significantly different (p<0.05).

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