Effects of Growth Factors and Gut Regulatory Peptides on Glucose Uptake 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 growth factors and gut regulatory peptides with insulin, *in vitro* experiment was conducted with HC11 cells. All the growth factor alone and the combinations of growth factors significantly ($p<0.05$) increased in glucose uptake. Insulin, EGF and IGF-1 exhibited a stimulation of glucose uptake for at least 24 h. Furthermore, the highest ($p<0.05$) synergistic effect was shown in EGF plus IGF-1 and the second synergistic effect in insulin plus EGF while no synergistic effect was found between insulin and IGF-1. However, the gut regulatory peptides neither potentiated nor inhibited the action of insulin on glucose uptake. Although growth factors did not modulates glucose uptake via increasing the rate of translation of the GLUT1 protein, RT-PCR analysis indicated that the growth factors significantly ($p<0.05$) increased the expression of GLUT1. The growth factors are therefore shown to be capable of modulating glucose uptake by transcription level with insulin in HC 11 cells. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 11 : 1690-1694)***

Key Words : Growth Factors, Gut Regulatory Peptides, Glucose Uptake, HC11, GLUT1

INTRODUCTION

An understanding of the regulation of glucose uptake by lactating mammary tissue is necessary to know the mechanism of milk production. Most researchers have concluded that insulin has no effect on glucose transport in isolated acini or mammary cells (Prosser and Topper, 1986). However, Threadgold and Kuhn (1984) reported the first evidence that glucose transport may be under the control of insulin in vivo in the rat mammary gland. Further support for a role of insulin comes from the observation that plasma insulin shows the expected transient rise after feeding in lactating rats (Mercer and Williamson 1986). Merlo et al. (1995) demonstrated that epidermal growth factor (EGF), IGF-1 and IGF-1 plus EGF induce DNA synthesis but have distinct effects on differentiation and survival in HC11 cells. It has also been known since the 1960's that glucose administrated orally is more effective in raising blood insulin levels than intravenous administration (Faulkner and Peaker, 1987). This provided evidence for a role of the gut in nutrient partitioning and initiated a search for substances derived from the gut which could promote insulin secretion (Choi et al., 2002). These substances were referred to as incretins. The first incretin isolated was called glucose-dependent insulinotropic polypeptide (GIP). Another incretin, glucagon-like polypeptide-1 (GLP-1), was isolated in the 1980's. However, although some growth factors have been known to play a crucial roles in mammary cell growth and proliferations, the study of changes on glucose uptake during the event of cell growth by some growth factors and gut regulatory peptides have not been well defined yet except for Wang et al. (1997) who concluded that GLP-1 is the first gut hormone shown to be cable of modulate glucose transporter levels in 3T3-L1 adipocytes. Thus, it was my intention to characterize the mode of growth factors and gut regulatory peptides on glucose uptake in the presence of insulin in HC11 cell line on molecular level.

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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. Medium was changed every 2 days. Insulin, epidermal growth factor (EGF), IGF-1, glucagon like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and somatotropin releasing inhibitory factor (SRIF) treatments were carried out as described in the figure legends.

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 Science Products, Inc.,) uptake. Briefly, HC11 cells were grown in 24-well culture plates. After 24 h pretreatment with insulin, growth factors or gut regulatory peptides or both together in starved (DMEM without glucose, Gibco BRL, # 23800), 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

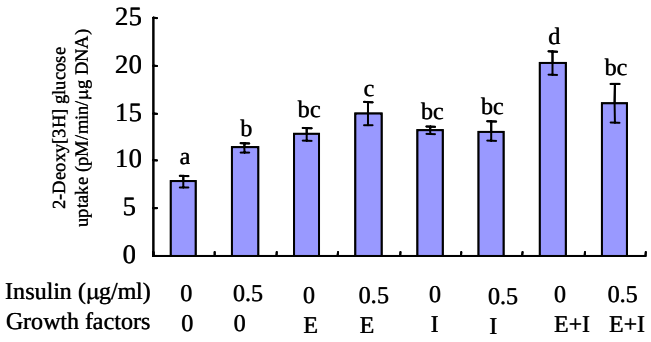

Figure 1. Effect of insulin and growth factors on glucose uptake of HC 11 cells. HC11 cells were cultured for 24 h in the presence or absence of various combination of insulin and/or growth factors (E; EGF 0.1 µg/ml, I; IGF-1 1.0 µg/ml, E+I; EGF 0.1 µ/ml+IGF-1 1.0 µg/ml). 2-Deoxy[3H]glucose uptake into cells was determined 30 minutes after exposure to starved media. Each value represents the mean±SEM of triplicate determinations from three experiments. a, b, c, d; means above the columns followed by different letters are significantly different ($p<0.05$).

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 1,218 Rackbeta Liquid Scintillation counter. To measure DNA, 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 24 h pretreatment with insulin and/or growth factors or gut regulatory peptides on 12 well culture plates, cells were washed twice with PBS and solubilized 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%, Tween 20, 1% polyvinylpyroiodide and 0.1% bovine serum albumin) and incubated overnight with polyclonal anti murine glucose transporter 1 (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% Tween 20, 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, pH 8.6 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 reverse transcription polymerase chain reaction (RT-PCR) analysis

After 24 h pretreatment with insulin and/or growth factors or gut regulatory peptides in 75 cm^2 flasks, total RNA was extracted by the acid/guanidium thiocyanate/ phenol chloroform method (Chomozynski and Sacchi, 1987). Reverse transcription was performed using the total RNA extracted in a reverse transcription mixture containing 50 mM Tris-HCl, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM dNTPs, 200 units M-MLV reverse transcriptase, 2 units/µl Rnase inhibitor and 2.5 µM oligo dT in a total volume of 25 µl at 42°C for 1 h. One µL of the first strand of cDNA was used as a template for PCR reaction with specific primers for GLUT1 and M. musculus 18S ribosomal RNA (18S). The oligonucleotides used as primers were: forward 5'-GAGAAGATGGCCACGGAGAGAG-3' and reverse 5'-GTCATCAACGCCCCACAGAA-3' for GLUT1 (William and Lee, 1991), and 5'-TACCTGGTTGA TCCTGCCAG-3' and 5'-CCAACTACGAGCTTTT TAAC-3' for 18S. Each reaction mixture consisted of 1 µL of cDNA, 20 pM of each primer, 1 µL of 10X Ex Taq buffer, 0.8 µL of dNTP mix, 0.5 units of Ex Taq polymerase and nuclease free water to a final volume of 50 µL. The reaction mixture was subjected to 20 cycles of denaturation (94°C, 30 sec), annealing (57.5°C for GLUT1, 62.0°C for 18S, 40 sec) and extension (72°C, 50 sec). After PCR amplification, the reaction mixture was run on 1% agarose gel in TE buffer. The gel was then stained with etidium bromide. The treatment differences were analyzed with a image analyzer (IR 1,500, Fujifilm, Japan).The 18S bands of etidium bromide was then used for normalization of RT-PCR GLUT1 expression levels.

Statistical analysis

Values presented in the figures are expressed as the ±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 growth factors : Figure 1 shows all the growth

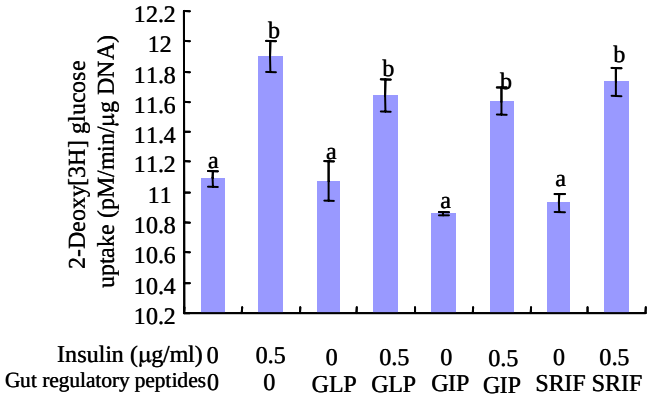

Figure 2. Effect of insulin and gut regulatory peptides on glucose uptake of HC 11 cells. On the day of confluent with growth media, medium was changed to quiescence media (QM) at 0.5 μg/ml of insulin and/or gut regulatory peptides (GLP; GLP-1 33 ng/ml, GIP; GIP 7.5 ng/ml, SRIF; SRIF 400 ng/ml) for 24 h. 2-Deoxy[3H]glucose uptake into cells was determined 30 minutes after exposure to starved media. Each value represents the mean ±SEM of triplicate determinations from three experiments.

factor alone and the combinations of growth factors significantly ($p<0.05$) increase in 2-Deoxy[3H]glucose uptake. Insulin, EGF and IGF-1 exhibited a stimulation of glucose uptake for at least 24 h. Furthermore, the highest ($p<0.05$) synergistic effect was shown in EGF plus IGF-1 and the second synergistic effect in insulin plus EGF while no synergistic effect was found between insulin and IGF-1. Bennett (1995) reported that insulin, IGF-1 and EGF exhibited a stimulation of glucose uptake activity of COMMA-D cells for at least 24 h, showing biphasic pattern. It was hypothesized that the initial stimulation up to three hours was due to the translocation of transporters from intracellular membranes to the cell surface and that the later response beginning after six hours was from de novo synthesis of new transporters arising from an increase in gene expression (Bennett, 1995). Even though some peptide factors such as EGF, fibroblast growth factor, insulin, IGF-1, platelet derived growth factor and stem cell factor stimulate growth, differentiation and survival of HC11 cell (Merlo et al., 1996), there has been no report on the glucose uptake of the cells with the growth factors so far. EGF and IGF-1 must have higher effect on glucose uptake in HC 11 cells than insulin as in Figure 1.

Effects of gut regulatory peptides

As can be seen from the results in Figure 2, in general, the gut regulatory peptides neither potentiated nor inhibited the action of 0.5 μg/ml insulin action on 2-Deoxy [3H]glucose uptake. Comparing to control group, GLP-1, GIP or SRIF alone also did not show any effect on 2-Deoxy [3H]glucose uptake in HC11 cells. The above results were almost same as those of Roe et al. (1995) who reported that

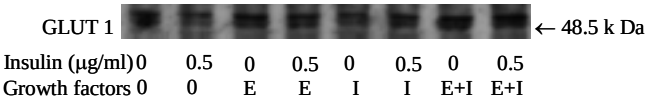

Figure 3. Effect of insulin and growth factors on GLUT1 protein content in HC 11 cells. HC11 cells were cultured for 24 h in the presence or absence of various combination of insulin and/or growth factors (E; EGF 0.1 μg/ml, I; IGF-1 1.0 μg/ml, E+I; EGF 0.1 μ/ml+IGF-1 1.0 μg/ml). HC 11 cell protein preparations (15 μg) were analyzed by Western blotting for the presence of GLUT1.

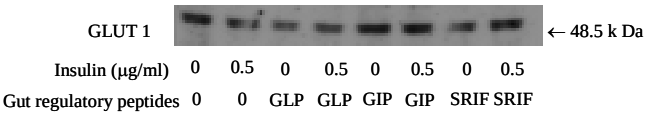

Figure 4. Effect of insulin and gut regulatory peptides on GLUT1 protein content in HC 11 cells. HC11 cells were cultured for 24 h in the presence or absence of various combination of insulin and/or gut regulatory peptides (GLP; GLP-1 33 ng/ml, GIP; GIP 7.5 ng/ml, SRIF; SRIF 400 ng/ml). HC 11 cell protein preparations (15 μg) were analyzed by Western blotting for the presence of GLUT1.

GIP and SRIF had no action in either the absence or presence of insulin with muscle cell cultures. On the other hand, enhanced insulin secretion with GIP and GLP-1 have been found to directly affect nutrient uptake in some tissues, due probably to identified receptors (Usdin et al., 1993). However, no receptors in mammary tissue have been reported yet.

Effects of growth factors and gut regulatory peptides on GLUT1 total protein level

We investigated further the effects of 24 h exposure of HC 11 cells to either growth factors and gut regulatory peptides or insulin, or both together, by measuring the total cellular content of GLUT1 protein. Western blot analyses from representative experiments are shown in Figures 3 and 4 respectively. These results suggest that gut growth factors and gut regulatory peptides with or without insulin did not modulate glucose uptake via increasing the rate of translation or the protein stability of the transporter.

Effects of growth factors and gut regulatory peptides on the expression of RT-PCR GLUT1

RT-PCR analysis indicated a 50% increase ($p<0.05$) in the expression of GLUT1 by insulin in Figure 5. There was also significant increases ($p<0.05$) with EGF alone and EGF plus insulin. While 1.0 μg/ml IGF-1 caused one and a half fold increase in GLUT1 expression levels, there was no synergistic effect ($p<0.05$) on insulin. On the other hand, compared with either EGF or IGF-1 alone, a 250% increase was found in EGF plus IGF-1 treatment. Compared to the EGF plus IGF-1 treatment, however, there was a significant

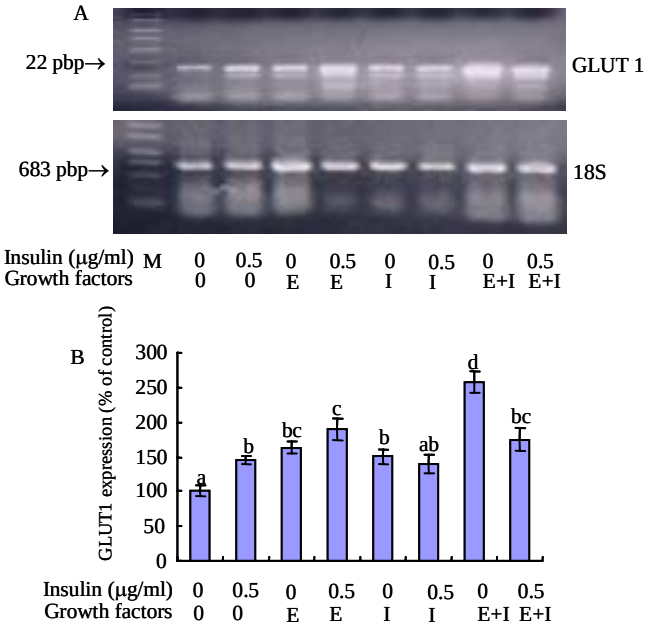

Figure 5. Effect of insulin and growth factors on RT-PCR GLUT1 expression of HC 11 cells. HC11 cells were cultured for 24 h in the presence or absence of various combination of insulin and/or growth factors (E; EGF 0.1 μg/ml, I; IGF-1 1.0 μg/ml, E+I; EGF 0.1 μg/ml+IGF-1 1.0 μg/ml). The lane marked M is PCR marker. The RT-PCR bands of M. musculus 18S ribosomal RNA (18S) was used for normalization of GLUT1 expression.
(A) The bands represent one individual experiment while (B) the bar graph indicates the mean±SEM of three independent RT-PCR analyses. a, b, c, d; means above the columns followed by different letters are significantly different ($p<0.05$).

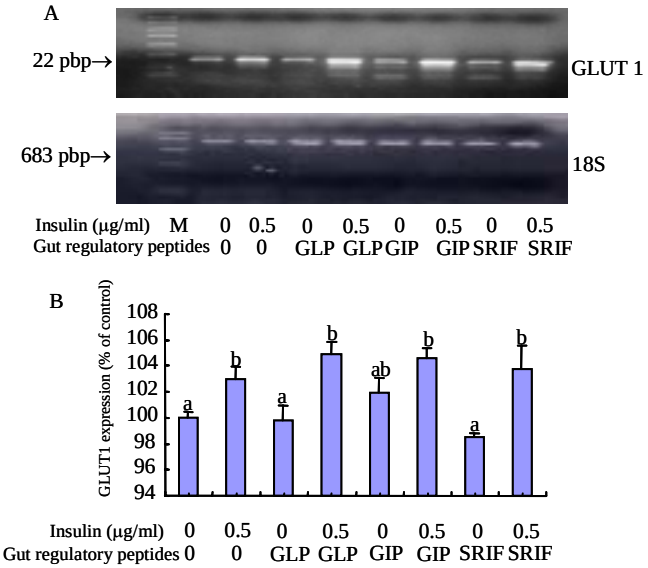

Figure 6. Effect of insulin and gut regulatory peptides on RT-PCR GLUT1 expression of HC 11 cells. HC11 cells were cultured for 24 h in the presence or absence of various combination of insulin and/or gut regulatory peptides (GLP; GLP-1 33 ng/ml, GIP; GIP 7.5 ng/ml, SRIF; SRIF 400 ng/ml). The lane marked M is PCR marker. The RT-PCR bands of M. musculus 18S ribosomal RNA (18S) was used for normalization of GLUT1 expression.
(A) The bands represent one individual experiment while (B) the bar graph indicates the mean±SEM of three independent RT-PCR analyses. a, b, c, d; means above the columns followed by different letters are significantly different ($p<0.05$).

($p<0.05$) decrease with the EGF plus IGF-1 and insulin. Therefore, it seems to be found that there is some toxic effect on glucose uptake in HC 11 cells treated with the combination of insulin, EGF and IGF-1 altogether. This increase in expression of GLUT1 was in relatively good agreement with the increase in transport activity measured in 24 h in insulin and/or growth factors as in Figure 1. 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 expression levels was induced by insulin and/or growth factors in HC 11 cells. This finding also clarifies that some growth factors alone or a combination of growth factors stimulate mammary glucose uptake in the presence of insulin (Page, 1989) in molecular level. I investigated further the effects of 24 h exposure of HC 11 cells to either insulin or GLP-1, GIP, SRIF, or both together, by measuring the RT-PCR expression level of GLUT1 as in Figure 6. Consistent with published data, treatment with insulin significantly increased the levels of GLUT1. No difference was observed between control cells and cells treated with 3 gut regulatory peptides alone. These results are in good agreement with those from glucose uptake (Figure 2). On the other hand these determination quite different from Wang et al. (1997) who suggested that GLP-1 modulates insulin action via increasing the rate of translation or the protein stability of the glucose transporters in 3T3-L1 adipocytes. So, it could be possible that the modulation of gut regulatory peptides with insulin in mammary cell, HC 11 is different from that of adipocytes. My data indicated that gut regulatory peptides with or without insulin has no ability to modulate glucose uptake in HC11 cells.

REFERENCES

Bennett, B. L. 1995. Glucose transporter gene expression in mammary tissue. Ph. D. Thesis, Univ.of Otago, New Zealand.

Bernier, M. D., M. Laird and M. D. Lane. 1988. Effects of vanadate on the cellular accumulation of pp15, an apparant product of insulin receptor tyrosine kinase action. J. Biol. Chem. 263:13626-13634.

Cesarone, C. F., C. Bolognesi and M. Peaker. 1980. Improved microflurorometric DNA determination n biological material using 33258 Hoescht. Anal. Boichem. 100:188-197.

Choi, B. H., K. W. Stewart, S. R. Davis and K. H. Myung. 2002. Bioluminescent determination of lactose secretion: A Measures of the in vitro performance of mammary acini from lactating

rats. Asian-Aust. J. Anim. Sci. 15:274-278.

Chomozynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-choloform extraction. Anal. Biochem. 162:156-159.

Doppler, W., B. Groner and R. K. Ball. 1989. Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat beta-casein gene promoter constructs in a mammary epithelial ell line. Proc. Natl. Acad. Sci. USA. 86:104-108.

Faulkner, A. and M. Peaker. 1987. Regulation of mammary glucose metabolism in lactation. New York, Plenum Press.

Laemmli, U. K. 1970. Cleveage of structural proteins during the assembled of the head of bacteriophage T4. Nature. 227:680-685.

Linzell, J. L. 1967. The effect of very frequent milking and of oxytocin on the yield and composition of milk in fed and fasted goats. J. Physiol. 190:333-346.

Mercer, S. W. and D. H. Williamson. 1986. Time course of changes in plasma glucose and insulin concentrations and mammary gland lipogenesis during refeeding of starved conscious lactating rats. Biochem. J. 239:489-492.

Merlo, G. R., D. Graus-Porta, N. Cella, B. M. Marte, D. Taverna and N. E. Hynes. 1996. Growth, differentiation and survival of HC11 mammary epitherial cells: diverse effects of receptor tyrosine kinase-activationg peptide growth factors. European J. Cell Biol. 70:97-105.

Merlo, G. R., L. Fiore, F. Basolo, L. Duboc and N. E. Hynes. 1995. P53 dependent and p53 independent apoptosis in mammary epitherial cells reveals a role for EGF and insulin as survival factors. J. Cell Biol. 128:1185-1196.

Page, T. 1989. Evidence for the involvement of a gastrointestinal peptide in the regulation of glucose uptake in the mammary gland of the lactating rat. Biochem. J. 258:39-643.

Roe, J. A. A. S. Haji Bara, J. M. M. Harper and P. J. Buttery. 1995. Effects of growth factors and gut regulatory peptides on nutrient uptake in ovine muscle cell cultures. Comp. Biochem. Physiol. 110A:107-114.

SAS Institute Inc. 1997. SAS Users Guide: Release 6.07. Cary, North Carolina, SAS Institute.

Threadgold, L. C. and N. J. Kuhn. 1984. Monosaccharide transport in the mammary gland of intact lactating rat. Biochem. J. 218:213-219.

Usdin, T. B., E. Mezey, D. C. Button, M. J. Brownstein and T. I. Bonner. 1993. Gastric inhibitory polypeptide receptor, a member of the secretin-vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs and the brain. Endocrinology. 133:2861-2870.

Wang, Y., H. K. Kole, C. Montrose-Rafizadeh, R. Perfetti, M. Bernier and J. M. Egan. 1997. regulation of glucose transporters and hexose uptake in 3T3-L1 adipocyte: glucogan-like peptide-1 and insulin interactions. J. Mol. Endocrinol. 19:241-248.

William, I. S. and E. C. Lee. 1991. Assessment of glucose transporter gene expression using polymerase chain reaction. Endocrinology, 128:2387-2394.