GnRH increases glucose transporter-1 expression and stimulates glucose uptake in the gonadotroph

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Abstract

GnRH is the main regulator of the hypothalamic–pituitary–gonadal (H–P–G) axis. GnRH stimulates the pituitary gonadotroph to synthesize and secrete gonadotrophins (LH and FSH), and this effect of GnRH is dependent on the availability of glucose and other nutrients. Little is known about whether GnRH regulates glucose metabolism in the gonadotroph. This study examined the regulation of glucose transporters (*Gluts*) by GnRH in the LβT2 gonadotroph cell line. Using real-time PCR analysis, the expression of *Glut1*, -2, -4, and -8 was detected, but *Glut1* mRNA expression level was more abundant than the mRNA expression levels of *Glut2*, -4, and -8. After the treatment of LβT2 cells with GnRH, *Glut1* mRNA expression was markedly induced, but there was no GnRH-induction of

Glut2, -4, or -8 mRNA expression in LβT2 cells. The effect of GnRH on *Glut1* mRNA expression is partly mediated by ERK activation. GnRH increased GLUT1 protein and stimulated GLUT1 translocation to the cell surface of LβT2 cells. Glucose uptake assays were performed in LβT2 cells and showed that GnRH stimulates glucose uptake in the gonadotroph. Finally, exogenous treatment of mice with GnRH increased the expression of *Glut1* but not the expression of *Glut2*, -4, or -8 in the pituitary. Therefore, regulation of glucose metabolism by GnRH via changes in *Gluts* expression and subcellular location in the pituitary gonadotroph reveals a novel response of the gonadotroph to GnRH.

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Introduction

GnRH is the master regulator of the hypothalamic–pituitary–gonadal (H–P–G) reproductive axis. It binds to receptors in the pituitary gonadotroph cell to increase the synthesis and secretion of LH and FSH, both of which stimulate the ovary and testis to release sex steroids and induce oocyte maturation and spermatogenesis, respectively. Glucose is an important fuel for hypothalamic regulation of the reproductive axis. It is well known that across species a reduction in glucose availability by caloric restriction or fasting impairs LH secretion, and subsequent treatment with glucose or food restores pulsatile LH secretion in different species (Foster & Olster 1985, Cagampang *et al.* 1991, Cameron *et al.* 1991, Schreihöfer *et al.* 1993, 1996, Murahashi *et al.* 1996, Nagatani *et al.* 1996, 2000). Additional studies showed that impairing glucose availability by infusing 2-deoxy-D-glucose or insulin in ovariectomized goats decreased GnRH pulse frequency, but subsequent infusion of glucose together with insulin restored normal GnRH pulse frequency (Ohkura *et al.* 2004).

In addition, a study using inhibitors of glycolysis showed that GnRH-stimulated release of LH was impaired (Sen *et al.* 1979, Howland 1980). There is limited information about glucose effects and mediators of glucose sensing in the pituitary gonadotroph. One study showed that acute hyperglycemia impaired GnRH-stimulated LH secretion in primary cultured porcine pituitary cells (Barb *et al.* 1995). Another study showed that fasting rats for 24 h reduced the expression of LH mRNA and other pituitary hormones as well as pituitary leptin expression (Crane *et al.* 2007). When fasted rats were given 10% glucose water for 24 h, there was restoration of both glucose and LH levels as well as pituitary leptin. In addition, treatment of fasted rats with leptin was shown to increase LH levels indicating that leptin may be important for glucose sensing in the gonadotroph. Together, these studies show that glucose is an important energy fuel required for normal regulation of the reproductive axis at both the level of hypothalamic GnRH neurons and the pituitary gonadotroph. Still further studies are needed to understand components of glucose sensing in

the gonadotroph and whether GnRH regulates glucose metabolism in the gonadotroph.

Glucose enters cells via the solute carrier family-2 (SLC-2) proteins, which are also commonly referred to as glucose transporters (GLUTs; Mueckler 1994, Uldry & Thorens 2004). There are over 13 GLUTs, which share a common overall structure but differ in their primary sequences, tissue expression, and subcellular localization. Some GLUTs have well characterized roles in glucose metabolism in specific tissues. In muscle and adipose tissues, insulin stimulates glucose uptake by inducing translocation of GLUT4 from intracellular compartment to the plasma membrane (Hajdich *et al.* 1995, Haney *et al.* 1995, Kandror *et al.* 1995a,b, Marsh *et al.* 1995). In the pancreatic β -cell, GLUT2 is important for glucose-stimulated insulin secretion (Mueckler 1994, Stenbit *et al.* 1997, Guillaum *et al.* 2000, Uldry & Thorens 2004). GLUT1, which is ubiquitously expressed, is primarily responsible for basal glucose uptake in most tissues especially in endothelial cells of the blood–brain barrier (Maher *et al.* 1994). In cardiac muscle cells, insulin modulates glucose metabolism by inducing GLUT4 and GLUT1-translocation (Rett *et al.* 1996, Egert *et al.* 1999). The role of GLUTs in the reproductive axis has been examined in the ovary and endometrium. In the oocyte and granulosa cell, GLUT1 is regulated by IGF1 and estradiol (Zhou *et al.* 2000). In the endometrial stromal cells, *Glut1* expression is regulated by estradiol and progesterone (Frolova *et al.* 2009). There is very limited information about the expression of *Gluts* in the pituitary gonadotroph. Microarray analysis of GnRH-stimulated gene expression in L β T2 gonadotroph cells detected an increase in *Glut1* expression but not other *Gluts* (Wurmbach *et al.* 2001, Mazhawidza *et al.* 2006). In this study, we report for the first time that several *Gluts* are expressed in L β T2 gonadotroph cells, but GnRH markedly induces *Glut1* expression and translocation to the plasma membrane and increases glucose uptake. GnRH induction of *Glut1* expression and glucose utilization in the pituitary gonadotroph provides new insights into the coordinated regulation of reproductive function and glucose metabolism by GnRH.

Materials and Methods

Reagents

GnRH and actinomycin D were purchased from Sigma–Aldrich. Cell culture media were purchased from Mediatech, Inc. (Manassas, VA, USA) and supplemented with penicillin–streptomycin solution (Hyclone Laboratories, South Logan, UT, USA) and 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA), Matrigel (BD Biosciences, Bedford, MA, USA). Other reagents for real-time PCR are as follows: DNase I (Invitrogen, Co.), High Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Foster City, CA, USA), TRIzol (Invitrogen Co.); Taqman

Gene Expression Assays (Applied Biosystems), other molecular biology reagents are as follows: DMSO (Mediatech, Inc.), Ethanol (Sigma–Aldrich), U0126 (Bis[amino[(2-aminophenyl)thio]methylene]butanedinitrile (Invitrogen, Co.), PBS (Fisher Scientific, Fair Lawn, NJ, USA), SDS (Fisher Scientific), Nonidet P40 (Calbiochem, LaJolla, CA, USA), Complete Protease Inhibitor cocktail (Roche Diagnostics), polyclonal rabbit GLUT1 and -8 IgG (Abcam, Inc., Cambridge, MA, USA), GAPDH (Chemicon–Millipore Co., Danvers, MA, USA), polyclonal rabbit α Tubulin IgG and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-mouse IgG-HRP (Chemicon–Millipore Co.), paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), rabbit isotype IgG (Invitrogen, Co.), SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA), GLUT1 peptide (Thermo Scientific), Alexa Fluor 633 goat-anti-rabbit (Invitrogen Co.), 4',6-diamidino-2-phenylindole (DAPI; Invitrogen Co.), Coomassie Plus Protein Assay Reagent (Thermo Scientific), Cytochalasin B (Sigma–Aldrich), with 2-deoxy-D-[3H]-glucose (Perkin–Elmer, Waltham, MA, USA), other reagents including calcium chloride, magnesium sulfate, HEPES, Tris base, sodium chloride, SDS, potassium chloride, and Tween 20, methanol, ethanol, Triton-X100, BSA-fraction V were purchased from Fisher Scientific, and LH EIA Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Animals

Female and male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). They were housed four or ten mice per cage, maintained on a 14:10 light–dark cycles and allowed *ad libitum* access to food and water. Prepubertal mice (3- to 4-week-old) were given exogenous GnRH 400 ng/kg (LHRH Sigma) or vehicle (0.9% NaCl) via i.p. injection as previously described (Chappell *et al.* 1999). Approximately 60–90 min after GnRH administration, mice were killed by isoflurane inhalation followed by cervical dislocation. Blood was then collected via cardiac puncture to measure hormone levels, and the pituitary gland was isolated for RNA extraction as described below to measure changes in gene expression. All protocols were conducted in accord with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the Committees of Animal Care and Use of Wayne State University.

LH assay

Blood collected from mice was treated with GnRH or vehicle and centrifuged at 12 000 *g* for 15 min, and then serum was transferred to clean tubes and stored at -20°C until used in assay. LH EIA Kit was used to measure LH in serum (50 μl of serum/mouse sample) from mice treated with vehicle or exogenous GnRH according to assay protocol by measuring

the optical density (OD) at 450 nm of serum samples and LH standards provided in kit. LH serum concentrations (mIU/ml) in samples were determined using ReaderFit (software at www.Readerfit.com) to plot OD vs concentration for standards. Fold effects were determined by normalizing GnRH-induced LH levels to vehicle-induced LH levels.

GnRH stimulation studies

LβT2 cells (Thomas *et al.* 1996, Turgeon *et al.* 1996) (approximately at 75% confluency or 1×10^6 cells/well in a 6-well plate) were grown in media containing 4.5 g/l glucose with sodium pyruvate supplemented penicillin–streptomycin solution, and 10% fetal bovine serum, and then treated with 10 nM GnRH for 2, 4, and 8 h. To perform dose–response studies, LβT2 cells were stimulated with GnRH (0.1–100 nM) for 2 h, and total RNA was isolated using TRIzol Reagent. Total RNA (1 µg) was treated with DNase I for time course and dose–response studies, and then cDNA was synthesized using the High Capacity cDNA Reverse Transcript Kit.

To perform studies with inhibitors, LβT2 cells were treated with 10 µg/ml of actinomycin D or DMSO as its vehicle control 30 min prior to the addition of GnRH (10 nM). Two hours after the addition of GnRH, total RNA from cells was prepared using the TRIzol Reagent and total RNA (2 µg) was used to synthesize cDNA as described above. For MAPK inhibitor studies, 10 µM U0126 [Bis[amino[(2-aminophenyl)thio]methylene]butanedinitrile] was added to cells 1 h prior to the addition of GnRH (10 nM). Two hours after the addition of GnRH, total RNA from cells was prepared using the TRIzol Reagent and total RNA (2 µg) was used to synthesize cDNA as described above.

Real-time PCR quantification of Gluts

The cDNA (representing 50 or 100 ng of starting RNA) was subsequently used for real-time PCR analyses via Taqman Gene Expression Assays for *Glut1* (Mn00441473_m1), *Glut2* (Mm00446224_m1), *Glut4* (Mm01245502_m1), *Glut8* (Mn00444634_m1), and *Tbp* (Mm00446973_m1). PCRs were conducted using the StepOne Plus Real-time PCR System (Applied Biosystems). Serial 10-fold dilutions of a representative sample were used initially to assess that the efficiency of each Taqman gene assay was 90–110%. Cycle threshold (C_t) was obtained for each sample. A corrected C_t (ΔC_t) was calculated by subtracting the *Tbp* C_t from the target genes (*Glut1*, -2, -4, and -8) C_t for each sample. Relative differences from the control sample were then calculated by using the formula: fold change = $2^{-(\text{Control } \Delta C_t - \text{Sample } \Delta C_t)}$.

Western blot analysis

Whole cell extracts were prepared from LβT2 cells treated with vehicle or GnRH (30 nM) for 2, 4, and 8 h using RIPA

buffer containing 1× PBS, 0.1% SDS, and 1% Nonidet P40 with 1× Complete Protease Inhibitor cocktail. Protein concentration was determined by using Coomassie Plus Protein Assay Reagent, and western blot analysis was performed on protein extracts (50 µg/lane) with a polyclonal antibody against GLUT1, -8, GAPDH, and αTubulin were used. Immunodetection of protein was performed using SuperSignal West Pico chemiluminescent reagent. Using the AlphaEase FC software Program, densitometric analysis was performed on autoradiographs of scanned images from western blot analysis of three sets of protein extracts for each time point (no GnRH and GnRH at 2, 4, and 8 h).

Immunostaining of LβT2 cells and confocal microscopy

LβT2 cells were grown on Matrigel-coated cover slips and stimulated with GnRH (100 nM) for 2, 4, and 8 h. Cells were fixed with 2% paraformaldehyde for 30 min and methanol–ethanol (50–50), and then permeabilized with 0.5% Triton-X for 10 min prior to blocking (10% BSA) for 60 min at room temperature (RT). Primary antibodies, rabbit-polyclonal GLUT1, and rabbit isotype IgG diluted 1:200 were applied overnight at 4 °C, while secondary antibody and Alexa Fluor 633 goat-anti-rabbit diluted 1:500 were incubated for 1 h the next day. After washing with PBS, mounting media with DAPI (a nuclear marker) was added to coverslips prior to mounting on slides. Slides were viewed with the Leica TCS SP5 Laser-scanning confocal microscope viewed at 63× magnification with oil using software LAS_AF version 2.3.1.

Glucose uptake assays

LβT2 cells were grown in normal media (4.5 g/l glucose with sodium pyruvate) supplemented with antibiotics and 10% fetal bovine serum in 24-well plates overnight. The next morning media were replenished and cells were stimulated with GnRH (100 nM) for 4 h. Cytochalasin B at 20 µM, an inhibitor of glucose transport (Lu *et al.* 1997), was added 30 min prior to glucose uptake analysis in control cells and cells were treated with GnRH. Glucose uptake assays were performed with 2-deoxy-D-[3H]-glucose radioisotopic technique as previously described (Kozma *et al.* 1993). GnRH-treated cells and controls were aspirated, washed, and incubated with HEPES-buffered solution (50 mM HEPES, pH 7.4, 136 mM sodium chloride 4.7 mM potassium chloride, 1.25 mM magnesium sulfate, and 1.25 mM calcium chloride) containing 0.5 µCi/ml 2-deoxy-D-[3H]-glucose for 10 min. Glucose transport was stopped by washing three times with ice-cold PBS. Cells were lysed with 1% SDS, and cell-associated radioactivity was determined by scintillation counting. To determine the specific glucose uptake, counts per minute (CPM) in the presence of cytochalasin B was subtracted from each corresponding time point and then normalized for DNA content. GnRH-stimulated fold effects were calculated by dividing GnRH-stimulated glucose uptake by basal glucose uptake (in the absence of GnRH).

Statistical analysis

Each data point represents the mean value $\pm$ S.E.M. of at least three measurements obtained in three or more repeated studies with the same experimental conditions. Significance was determined at $P < 0.05$ by analyzing data using ANOVA followed by Bonferroni *post hoc* test or Student's *t*-test analysis using Prism (GraphPad Software, Inc., San Diego, CA, USA) or Mann–Whitney *U* test.

Results

Gluts are expressed in L β T2 gonadotroph cells and murine pituitary

Since others have shown by microarray analysis that GnRH increases *Glut1* expression (Wurmbach *et al.* 2001, Mazhawidza *et al.* 2006), we compared the relative basal expression of *Glut1* to the basal expression of other *Gluts* in L β T2 gonadotroph cells using real-time PCR analysis. The basal expression of *Glut1* was 1.34-fold, which was

significantly higher than the basal expression of *Glut2* (0.1-fold), *Glut4* (0.2-fold), and *Glut8* (0.7-fold) (Fig. 1A, $P < 0.001$ for *Glut1* vs *Glut2* and $P < 0.01$ for *Glut1* vs *Glut8*). The basal expression of *Glut8* was significantly higher than the basal expression of *Glut2* (Fig. 1A, $P < 0.05$).

We next investigated whether GnRH regulates *Glut1* and other *Gluts* (*Glut2*, -4 , and -8) mRNA expression in L β T2 cells. Using real-time PCR analysis, we found that GnRH increased *Glut1* mRNA expression 19.4-fold at 2 h, 18.8-fold at 4 h, and 8.3-fold at 8 h, respectively (Fig. 1B, $P < 0.05$ no GnRH (0 h) vs GnRH at 2 h and $P < 0.05$ no GnRH (0 h) vs GnRH at 4 h). In contrast, GnRH decreased *Glut4* mRNA expression (Fig. 1B, $P < 0.05$, no GnRH (0 h) vs GnRH at 8 h). GnRH had no significant effect on *Glut2* or -8 mRNA expression (Fig. 1B). Since the peak effect of GnRH on *Glut1* expression occurred with GnRH treatment at 2 h, dose–response studies were performed with increasing concentrations of GnRH (0.1–100 nM) at 2 h. *Glut1* mRNA expression increased 3.3-fold at 0.1 nM, 11.8-fold at 1 nM, 14.6-fold at 10 nM, 16.8-fold at 30 nM, and 18.8-fold at 100 nM GnRH (Fig. 1B, $P < 0.05$ no GnRH vs GnRH at 10 nM and $P < 0.01$, no GnRH vs GnRH at 30 or 100 nM). GnRH dose–response studies showed no significant changes in the expression of *Glut2*, -4 , or -8 mRNA levels (Fig. 1B).

To further investigate mechanism by which GnRH increases *Glut1* mRNA expression additional studies were performed with inhibitors of transcription and MAPK

Figure 1 Regulation of glucose transporters by GnRH in L β T2 cells. (A) RNA was isolated from L β T2 cells and real-time PCR analysis was performed. Basal expression of *Glut1*, -2 , -4 , and -8 was normalized to basal expression of *Tbp* and then fold effects were determined by comparing basal expression of *Glut1* (white bar), *Glut2* (light gray bar), *Glut4* (dark gray bar), and *Glut8* (black bar) to the basal expression of *Glut1*. Each bar represents the mean $\pm$ S.E.M. of six experiments (and each experiment contained three samples that were also averaged) as shown in the graph. (B) L β T2 cells were stimulated with GnRH (10 M⁻⁹) for 2–8 h and total RNA was isolated to make cDNA and then subjected to real-time PCR analysis. GnRH-treated cells (2–8 h) were compared to untreated cells (0 h) and fold effects of *Glut1*, -2 , -4 , and -8 gene expression were determined after normalization with *Tbp* expression. L β T2 cells were stimulated with GnRH (0.1–100 M⁻⁹) for 2 h and total RNA was isolated to make cDNA and then subjected to real-time PCR analysis. Fold effects of *Glut1*, -2 , -4 , and -8 gene expression in GnRH-treated cells were compared to untreated cells and were determined after normalization with *Tbp* expression. (C) L β T2 cells were treated with treated with actinomycin D (10 μ g/ml) 30 min prior to adding GnRH (10 M⁻⁹). Two hours after the addition of GnRH, total RNA was isolated to make cDNA, which was then subjected to real-time PCR analysis. After normalization with *Tbp* expression, fold effects were determined by comparing *Glut1* mRNA expression induced by GnRH (+/– actinomycin D) to *Glut1* mRNA expression in cells in the absence of GnRH or actinomycin D. Each graph contains bars that represent the mean $\pm$ S.E.M. of three experiments (and each experiment contained three samples per treatment condition that were also averaged). Significance was determined using ANOVA analysis with Bonferroni *post hoc* test * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$.

Figure 2 GnRH-stimulated *Glut1* mRNA expression requires transcriptional activation and ERK activation. (A) LβT2 cells were treated with U0126 (10 M^{-6}) 60 min prior to adding GnRH (10 M^{-9}). Two hours after the addition of GnRH, total RNA was isolated to make cDNA, which was then subjected to real-time PCR analysis. After normalization with Tbp expression, fold effects of *Glut1* expression were determined by comparing *Glut1* mRNA expression induced by GnRH +/- U0126 to the expression of *Glut1* in cells in the absence of GnRH or U0126. Each graph contains bars that represent the mean $\pm$ S.E.M. of three or more experiments (and each experiment contained three samples per treatment condition that were also averaged). Significance was determined using ANOVA analysis with Bonferroni *post hoc* test ** $P < 0.01$; *** $P < 0.001$. (B) LβT2 cells were treated with U0126 (10 M^{-6}) 60 min prior to adding GnRH (10 M^{-9}) for 10, 30, or 120 min and then whole extracts were made. Extracts were used in western blot analysis with phosphorylated ERK and ERK antibodies as shown. The autoradiograph of a representative experiment is shown.

signaling. Cells were pretreated with actinomycin D, an inhibitor of transcription, 30 min prior to the addition of GnRH (10 nM) and then 2 h later changes in *Glut1* mRNA expression were examined by real-time PCR analysis. As seen previously, *Glut1* mRNA expression was increased by 18.8-fold by GnRH alone (Fig. 1C, $P < 0.001$, untreated (no GnRH) vs GnRH treated). In the presence of actinomycin D, induction of *Glut1* expression by GnRH was completely blocked, but actinomycin D alone did not inhibit basal expression of *Glut1* mRNA (Fig. 1C, $P < 0.001$ GnRH without actinomycin D vs GnRH with actinomycin D).

We next investigated signaling pathways involved in mediating effects of GnRH on *Glut1* gene expression. GnRH is known to activate several MAPK pathways in gonadotroph cells (Kraus *et al.* 2001, Liu *et al.* 2002). We examined whether the signaling pathway that activates the ERK, one of the GnRH-activated MAPKs, is important for mediating effects of GnRH on *Glut1* mRNA gene expression. Pretreatment with U0126, an inhibitor of ERK activation, blocked GnRH-stimulated *Glut1* mRNA expression by 48% (Fig. 2A, $P < 0.01$, GnRH with vehicle vs GnRH with U0126) and blocked GnRH-induced phosphorylation of ERK (Fig. 2B).

GnRH increases GLUT1 protein in LβT2 cells

Since GnRH stimulates *Glut1* mRNA levels, the effect of GnRH on GLUTs protein content in the gonadotroph was examined. Western blot analysis was performed with whole cell extracts isolated from LβT2 cells treated with GnRH (30 nM). Several GLUT1 protein bands were detected between 37 and 50 kDa and these bands were not detected in the presence of excess GLUT1 peptide (data not shown). GnRH increased GLUT1 protein level by 3.5-fold at 4 h and 6.6-fold at 8 h (Fig. 3A, lane 1 vs lanes 2–4 and Fig. 3B, $P < 0.05$ no GnRH (0 h) vs GnRH at 8 h). The effect of GnRH on GLUT8 protein level was not statistically significant (Fig. 3A and B). Western blot analysis did not detect GLUT2 or -4 protein in LβT2 whole cell protein extracts in the absence or presence of GnRH treatment (data not shown).

To further examine the regulation of GLUT1 protein by GnRH, immunofluorescent analysis with confocal microscopy was performed to examine the subcellular location of GLUT1 protein in GnRH-treated LβT2 cells (Fig. 4). In the absence of GnRH, GLUT1 was diffusely distributed in the cytosol and perinuclear region of the cell (Fig. 4A, untreated cells, no GnRH). In the presence of GnRH, GLUT1 protein increased at the cell surface in a time-dependent manner that is clearly seen at 4–8 h after GnRH treatment (Fig. 4A, no GnRH vs 4B–D, GnRH 2–8 h). In contrast, GnRH showed a transient increase in GLUT8 protein in the perinuclear region of the cell (Fig. 4E, no GnRH vs 4F–H, GnRH 2–8 h). These results show that in LβT2 cells GnRH increases the detection of GLUT1 at the cell surface, indicating that GnRH stimulates GLUT1

Figure 3 GnRH increases GLUT1 protein expression in LβT2 cells. (A) Whole cell extracts (50 gm^{-6}) were prepared from LβT2 cells after stimulation with GnRH (30 M^{-9}) for 2 h (lane 2), 4 h (lane 3), and 8 h (lane 4) or no GnRH (lane 1) and western blot analysis was performed with GLUT1, -8, and GAPDH antibodies. A picture of a representative autoradiograph is shown. (B) Densitometry analysis of GnRH-induced GLUT1 and -8 protein content normalized for GAPDH protein content was performed with three sets of extracts for each time point and the mean $\pm$ S.E.M. is shown in graphs. Significance was determined using ANOVA with Bonferroni *post hoc* test. * $P < 0.05$ no GnRH (0 h) compared to GnRH at 8 h.

translocation in LβT2 cells. Competition studies with GLUT1 peptide blocked the detection of GLUT1 immunofluorescence (data not shown).

GnRH stimulates glucose uptake in LβT2 cells

Since GnRH increases GLUT1 protein levels and detection of GLUT1 at the cell surface, we investigated whether GnRH stimulates glucose uptake in LβT2 cells. Glucose uptake assays were performed with 2-deoxy-D-[3H]-glucose. LβT2 cells were grown in normal medium and then stimulated with GnRH (100 nM) for 4 h. Compared to basal glucose uptake, GnRH increased glucose uptake by 1.8-fold in LβT2 cells (Fig. 5, $P < 0.05$). This increase in glucose uptake corresponded to the time-dependent increased detection of GnRH-stimulated GLUT1 protein at the cell surface (Fig. 4C, 4 h GnRH). Therefore, GnRH stimulates glucose uptake in LβT2 cells.

GnRH regulates GLUT1 gene expression in the murine pituitary gland *in vivo*

Since our studies were conducted using the LβT2 cell line, we further investigated whether GnRH induced upregulation of *Glut1* expression in gonadotrophs *in vivo*. We treated 3- to 4-week-old C57BL/6J mice with exogenous GnRH (LHRH) or vehicle and then collected blood and the pituitary gland 60–90 min after injection of GnRH or vehicle. Serum LH levels were measured by ELISA. Total RNA was isolated from the pituitary gland to make cDNA to examine the expression of *Gluts* gene expression using real-time PCR analysis. GnRH treatment increased *Glut1* mRNA levels 2.6-fold in prepubertal mice compared to vehicle-treated

Glut1 levels (Fig. 6A, $P < 0.001$). There was no significant change in *Glut2*, -4, or -8 mRNA levels in GnRH-treated mice compared to vehicle-treated mice (Fig. 6A). Serum LH levels increased 5-fold in GnRH-treated mice compared to vehicle-treated mice (Fig. 6B, $P < 0.05$).

Discussion

Our study is the first to show that GnRH stimulates glucose uptake in the pituitary gonadotroph cells in LβT2 cells, which express several Gluts. This is a novel response of the gonadotroph cell to GnRH, which has not been previously examined. Others have shown that increased uptake of [¹⁸F]2-fluoro-2-deoxy-D-glucose in the pituitary gland of women was associated with estrogen-induced LH secretion (Ottowitz *et al.* 2008). Therefore, glucose uptake in the pituitary may serve as an important marker of energy metabolism that is important for the regulation of LH secretion.

Although others have shown that GnRH increases the expression of *Glut1* mRNA gene expression (Wurmbach *et al.* 2001, Mazhawidza *et al.* 2006), we have additional studies that have examined the regulation of other *Gluts* mRNA and protein expression in LβT2 gonadotroph cells. The activation of *Glut1* by GnRH is acute with a maximal activation obtained by 2 h followed by a reduction in *Glut1* mRNA by 8 h. Our studies were performed in serum containing media, which is known to rapidly degrade GnRH, and this may account for the decrease in *Glut* expression in long-term culture. In contrast, the expression of *Glut4* decreased after 8 h of GnRH treatment. GnRH had no significant effect on the expression of *Glut2* or -8 in LβT2

Figure 4 GnRH induces translocation of GLUT1 protein in LβT2 cells. LβT2 cells were grown on Matrigel-coated coverslips in normal media and stimulated with GnRH (100 M^{-9}) for 2–8 h. Immunofluorescent analysis was performed with GLUT1 antibody (A, no GnRH, –), (B, GnRH at 2 h), (C, GnRH at 4 h), (D, GnRH at 8 h) and GLUT8 antibody (E, no GnRH, –), (F, GnRH at 2 h), (G, GnRH at 4 h), and (H, GnRH at 8 h) to show subcellular localization of glucose transporters. All images show merged GLUT1 or -8 immunofluorescence with DAPI nuclear staining. Images of cells viewed at $63\times$ magnification with oil are shown.

Figure 5 GnRH stimulates glucose uptake in LβT2 cells. LβT2 cells were grown in normal media and stimulated with GnRH (100 M^{-9}) or vehicle (media alone) for 4 h. Glucose uptake assays were performed with 2-deoxy-D-[3H]-glucose (2-DOG). The graph represents the average of three experiments. In each experiment, there were four to six samples for control and four to six samples for GnRH. * $P < 0.05$ GnRH compared to control was determined by Mann–Whitney *U* test.

cells. Therefore, GnRH differentially regulates the expression of *Gluts* in LβT2 cells.

The regulation of *Glut1* gene expression by GnRH was further examined by using inhibitors of transcription and MAPK signaling. The acute increase in *Glut1* expression induced by GnRH in LβT2 cells was completely blocked by actinomycin D, an inhibitor of transcription. Therefore, GnRH stimulates *Glut1* mRNA by increasing gene transcription without affecting *Glut1* mRNA stability. These results are consistent with other reports that enhanced gene transcription is one mechanism by which *Glut1* expression is regulated (Kitagawa *et al.* 1991, Kozlovsky *et al.* 1997, Fong *et al.* 2004, Abdul Muneer *et al.* 2011). Our study also showed that induction of MAPK signaling is involved in mediating effects of GnRH on *Glut1* gene expression in LβT2 cells. Several studies have shown that ERK signaling is important for mediating effects of GnRH on gene expression and gonadotroph function (Roberson *et al.* 1995, Liu *et al.* 2002, Bliss *et al.* 2009, 2010). In addition, ERK activation is also important for the regulation of *Glut1* gene expression in different tissues (Montessuit & Thorburn 1999, Fong *et al.* 2001, Nose *et al.* 2003, Santalucia *et al.* 2003). In our study, the activation of *Glut1* gene expression by GnRH is partly dependent on MEK activation. Lack of complete inhibition of GnRH-stimulated *Glut1* expression by the MEK inhibitor U0126 suggests that other GnRH-induced signaling pathways are also involved in mediating effects of GnRH on *Glut1* gene expression in the gonadotroph.

To further examine the functional significance of GnRH-induced *Glut1* mRNA expression, we also examined the effect of GnRH on GLUT1 translocation. Our study shows that GnRH increases GLUT1 translocation to the cell surface. However, our study did not show that GnRH-induced translocation is independent of an effect of GnRH on *Glut1* transcription because our immunofluorescent studies

were not done in the presence of actinomycin D. Still, the increased detection of GLUT1 at the cell surface correlates with GnRH-stimulated glucose uptake. Although in most cells GLUT1 primarily mediates basal glucose uptake, studies have shown that in cardiac myocytes insulin stimulates translocation of GLUT1 to the plasma membrane and thus GLUT1 plays a role in insulin-stimulated glucose uptake in cardiac myocytes (Rett *et al.* 1996, Egert *et al.* 1999). Therefore, LβT2 gonadotroph cells mediate responses to GnRH that impact glucose uptake and translocation of GLUTs.

The LβT2 cell line, which was derived from a gonadotroph tumor that expresses SV40 T antigen, has served as a very useful *in vitro* model for examining the effects of GnRH and other hormones on pituitary gonadotroph function (Thomas *et al.* 1996, Turgeon *et al.* 1996). We have now shown that

Figure 6 GnRH stimulates *Glut1* expression in the mouse pituitary gonadotroph. (A) C57/Bl6 prepubertal male and female mice were given GnRH i.p. injection and 60–90 min later pituitary glands were collected. Total RNA was isolated from the pituitary gland and real-time PCR analysis was performed on pituitary RNA isolated from prepubertal vehicle-treated mice (15–16 mice) and prepubertal GnRH-treated mice (16–17 mice). Student's *t*-test was performed with *** indicating P value < 0.001 for GnRH-treated mice compared to vehicle-treated mice. (B) C57BL/6J prepubertal male and female mice were given GnRH i.p. injection, and blood was collected 60–90 min later. LH immunoassays were performed with serum from prepubertal vehicle-treated mice (four mice) and prepubertal GnRH-treated mice (eight mice). Student's *t*-test was performed with * indicating P value < 0.05 for GnRH-treated mice compared to vehicle-treated mice.

these cells can also serve as an *in vitro* model to investigate changes in glucose metabolism in the gonadotroph. We speculate that the regulation of glucose metabolism by GnRH in the gonadotroph may play an important role in the synthesis and secretion of gonadotrophins.

Our study also investigated the regulation of *Glut1* expression in the pituitary gland of prepubertal mice after treatment with exogenous GnRH. We used prepubertal mice because GnRH levels are known to increase after puberty. GnRH-induced *Glut1* mRNA upregulation in gonadotrophs occurs under conditions that also increased serum LH levels in both female and male mice. Therefore, this data provides *in vivo* support of results in LβT2 cells that GnRH regulates *Glut1* mRNA expression but not *Glut2*, -4, or -8 in the pituitary gonadotroph.

Finally, it is well known that disorders of pubertal development and infertility, e.g. precocious puberty and polycystic ovary syndrome, are associated with obesity and type 2 diabetes mellitus. The data from our study shows that GnRH increased glucose utilization in the gonadotroph, and this indicates that in the gonadotroph GnRH regulates both glucose metabolism and gonadotrophin synthesis and secretion. Therefore, this study provides a foundation to further investigate the role of glucose utilization in the gonadotroph which may provide new insights into our understanding of disorders of the reproductive axis that are associated with abnormal glucose metabolism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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