Estrogen Simulation of Glucose Transporter Expression in Endometrial Cancer Cells

Miyabi Saito

Research under the supervision of Jill Slack-Davis, in the University of Virginia Department of Microbiology, Immunology and Cancer Biology

Abstract

Type I endometrial cancer responds to estrogen and is highly associated with obesity. Estrogen levels are elevated in obese women, perhaps indicating a link between estrogen and obesity-associated endometrial cancer. Previous studies have shown that estrogen regulates expression and activity of proteins involved in glucose metabolism, including glucose transporters. Additionally, expression of the glucose transporter, GLUT6 was elevated in malignant endometrium of obese women compared to non-tumor tissue. Loss of GLUT6 expression in endometrial cancer cells resulted in cell death, indicating a potential role of GLUT6 in tumor growth. Importantly, the GLUT6 promoter contains estrogen response elements. Therefore, we evaluated whether estrogen regulates GLUT6 expression. Human endometrial cancer cells (MFE296) were stimulated with estrogen, and expression of GLUT1, which responds to estrogen and served as the positive control, and GLUT6, were examined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). As expected, GLUT1 expression increased with estrogen stimulation; however, there was no significant change in expression of GLUT6. These results indicate that GLUT6 expression does not parallel GLUT1 in response to estrogen in MFE296 cells. Future studies will be designed to test different concentrations of estrogen in additional endometrial cancer cell lines and to examine the effect of estrogen on GLUT6 protein to identify potential conditions of estrogen-dependent regulation.

Introduction

As one of the most common gynecological malignancies, endometrial cancer affects more than 287,000 women each year worldwide [1]. It is characterized by uncontrolled proliferation of the epithelial cells lining the uterus, and left untreated, further progression involving metastasis to lymph nodes, liver, and lungs leads in death [2]. Endometrial carcinomas occur most frequently in postmenopausal women and can be classified into two types. While type II tumors (predominantly clear cell and papillary serous) are more invasive and estrogen/progesterone receptor (ER/PR) negative, meaning the endometrial cancer cells do not respond to either hormone, they account for less than 15% of all cases. On the other hand, the more prominent type I tumors (endometrioid) are less invasive, estrogen responsive (ER/PR positive), and associated with higher survival rates [3]. Data from women on hormone replacement therapy (HRT) indicated a significant increase in the incidence of endometrial cancer when estrogen was provided in the absence of progesterone, thus demonstrating the importance of estrogen to endometrial cancer growth and progression [4].

Type I endometrial cancer is more strongly associated with obesity than any other cancer, as up to 57% of endometrial cancers in the United States are linked to excessive adiposity [5]. Even after diagnosis, obesity affects outcomes as obese women have a 6-fold increased risk of mortality from the disease [6]. Estrogen, which stimulates proliferation of the endometrium, is elevated in obese women; however, it is unclear whether this increase, which is markedly less than that provided to women on HRT, is sufficient to drive endometrial cancer development or progression [5].

Obesity is also associated with poor serum glucose control with many patients suffering from metabolic syndrome or type-II diabetes. Interestingly, both type-I and type-II diabetes are associated with an increased risk of endometrial cancer [7]. Adequate endometrial glucose metabolism is important for normal endometrial cell proliferation and differentiation [8], and aerobic glycolysis, which requires excess glucose, is a hallmark of cancer [9]. Preliminary evaluation of serum and tissues from obese women with and without endometrial cancer showed highly elevated glucose concentrations and expression of genes regulating glucose transport and metabolism in malignant endometria, respectively [10].

A family of facilitative glucose transporters, GLUTs, selectively allows passive diffusion of substrate across the cellular membrane [11]. The human endometrium expresses the ubiquitously

expressed transporter, GLUT1 [11]. Strikingly, expression of a poorly characterized glucose transporter GLUT6 was increased in tumors from obese endometrial cancer patients [10]. Loss of GLUT6 expression following siRNA knockdown significantly increased endometrial cancer cell death and reduced glucose uptake [10]. These data suggest that endometrial cancer cells may become dependent on GLUT6 for glucose uptake, and that glucose metabolism is important for endometrial cancer cell proliferation.

Estrogen, specifically estradiol (E2) regulates expression and activity of proteins involved in glucose metabolism including glucose transporters [12]. Furthermore, the GLUT6 promoter contains estrogen response elements [data not shown]. Therefore, **we investigated whether E2 stimulates expression of GLUT6 to promote endometrial cancer cell growth and survival**.

Methods

Cell Culture and Estrogen Stimulation Human endometrial cancer cells (MFE296) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells (100,000 cells) were plated in each well of a 6- well plate, serum-starved (RPMI-1640 containing 1% FBS) overnight, and stimulated with 1 nM 17β-Estradiol (suspended in 20% ethanol, Sigma) and 2% FBS for 0.5, 1, 2 and 4 hrs. Serum-starved cells and those stimulated with vehicle (2% FBS-RPMI with 0.01% EtOH) served as controls.

qRTPCR Cells were harvested, and RNA was isolated using TRIzol reagent and phenol-chloroform separation techniques [13]. Following TURBO DNase (Thermo Fisher TURBO DNA-free kit) treatment, RNA (1 µg) was reverse transcribed into cDNA (Life Science iScript cDNA synthesis kit). ROX fluorescence was used to perform quantitative RT-PCR (qRT-PCR) using primers directed against GLUT1, GLUT6, glucose-6-phosphate dehydrogenase (GAPDH), β-Glucuronidase (GUS), and β-2-microglobulin (B2M). Cycle threshold values (C_t) were obtained from three independent experiments and used to 1) calculate $\Delta\Delta C_t$ or 2) directly determine amplified DNA concentrations by comparing to standard curves (see below).

$\Delta\Delta C_t$ analysis Cycle threshold (C_t) values for GLUT1 and GLUT6 were normalized to each of the house-keeping controls (GAPDH, GUS, B2M). The normalized values were then normalized to the vehicle to obtain the $\Delta\Delta C_t$ values. Fold differences in expression were calculated using the equation: $2^{-\Delta\Delta C_t}$. The values were averaged for the three independent experiments, and standard deviations were calculated.

Standard curve RNA from non-E2-treated MFE296 cells was isolated then reverse transcribed into cDNA using the previous procedures (see above). The cDNA was amplified using PCR with primers directed against GLUT1, GLUT6, and controls B2M and GUS. PCR products were electrophoresed through 2% agarose, gel-purified, quantified by Nanodrop, and used as standards. Equal concentrations of each standard were serially diluted 10-fold from 1 ng for 0.1, 0.01, 0.0001, 0.00001 ng, in H_2O and used to generate standard curves by qRT-PCR for each experiment. C_t values for each primer set were obtained for duplicate samples in each of three

independent experiments and plotted on the standard curve for each experiment to determine the PCR-amplified DNA concentrations, which were normalized to the vehicle control.

Statistical Analysis Data were analyzed using a one-way ANOVA test. For any data sets with α significance of 0.05, the student's t-test was used to determine significance of individual points relative to vehicle control.

Results

Estrogen, specifically estradiol (E2), stimulates expression of several genes that regulate cellular metabolism, including glucose transporters [11]. To determine whether E2 stimulates GLUT6 expression to promote endometrial cancer cell proliferation, MFE296 cells were stimulated, and expression of GLUT6 and GLUT1, a gene reported to be regulated by estrogen [14], was determined by qRT-PCR. Relative expression of GLUT1 and GLUT6 was normalized to GUS and GAPDH, which served as controls, using the $\Delta\Delta C_t$ approach. Using GUS as the normalizing control, peak GLUT1 expression (2.5 fold) was observed at 0.5 and 4 hours after E2 stimulation (Figure 1A). When normalized to GAPDH, GLUT1 expression did not change significantly over time. However, GAPDH expression can be stimulated by E2 treatment [15], which may account for the discrepancy between the two normalizations. GLUT6 expression did not change over time following E2 stimulation (Figure 1B) when normalized to either control.

These results were confirmed by directly quantifying gene expression by comparing experimental qRT-PCR values to standard curves generated from known concentrations of each template. The standard curve constructed from five 10-fold dilutions of non-E2 treated DNA fit well into logarithmic curves with high R^2 values (Figure 2). The C_t values from a representative experiment were fit onto each standard curve to calculate the amount of amplified DNA expressed for each time point, which was normalized to the vehicle control for each gene. With this approach, GLUT1 (Figure 3A) increased at 0.5, 2 and 4 hours, whereas GLUT6 (Figure 3B) increased at 0.5 hours. However, the controls B2M (Figure 3C) and GUS (Figure 3D) also fluctuated over time, raising question regarding the apparent stimulation of GLUT1 and GLUT6.

In order to account for the variation in control gene expression, the data for this experiment were also analyzed using the $\Delta\Delta C_t$ analysis method. GLUT1 (Figure 4A) normalized to B2M showed a 4-fold increase in gene expression at 0.5 hour and an 8-fold increase at 4 hours. Normalization to GUS showed a 3.5-fold increase at 0.5 hours, and a 5-fold increase in GLUT1 expression at 4 hours. The changes in expression at 2 hours were not significant. GLUT6 (Figure 4B) normalized to B2M and GUS showed no significant changes in expression.

Discussion

Estrogen stimulates growth of Type I endometrial cancer, and obese women have elevated levels of circulating estrogen [5]. Estrogen also stimulates gene expression of proteins that regulate cellular metabolism. Previous studies demonstrated that estrogen stimulates expression of the glucose transporter, GLUT1 [14]. Our data confirmed this finding and showed that estrogen was unable to increase expression of GLUT6, a second glucose transporter with elevated expression in endometrial cancers from obese women [10].

GLUT1 expression determined using the $\Delta\Delta C_t$ analysis varied depending on the housekeeping gene used for comparison; normalization to GUS and B2M showed an estrogen-dependent increase in expression, while normalization to GAPDH revealed no change in expression. However, estrogen has been reported to regulate GAPDH gene expression [15], further indicating a potential role for E2 in cellular metabolic regulation, and perhaps more importantly, underscoring the need to use multiple controls for PCR analysis. Moreover, directly measuring the amount of amplified DNA indicated an estrogen-dependent increase for each of the primer sequences tested (GLUT1, GLUT6, B2M and GUS) within 30 minutes. While this observation could be the result of experimental error, it was reproducible, raising the question of whether estrogen also regulates expression of GUS and B2M.

At this point, we are unable to determine definitively whether estrogen regulates GLUT6 expression in endometrial cancer, as the experiments were conducted in one endometrial cancer cell line, MFE296. It is important to consider whether estrogen stimulates GLUT6 expression in other endometrial cancer cell lines. Furthermore, the data evaluated the effect of estrogen stimulation on GLUT6 at a transcription level, as the E2 binding to its receptor activates gene transcription [16] and the GLUT6 promoter contains ER response elements [10]. However, it is possible that E2 indirectly regulates GLUT6 protein expression, stabilization or localization to promote endometrial cancer cell growth. Future studies will be designed to interrogate the effect of estrogen on GLUT6 protein expression by Western blot or its subcellular localization by immunofluorescence.

Figures

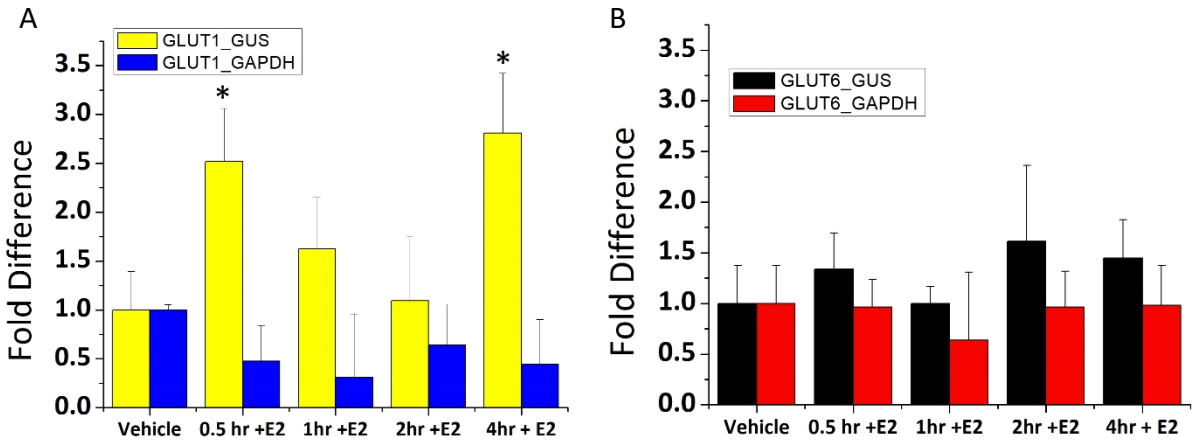

Figure 1. Fold differences in A) GLUT1 and B) GLUT6 gene expression following E2 stimulation for the indicated times, using GUS and GAPDH as controls. Data indicate the mean ± standard deviation of the $\Delta\Delta C_t$ values normalized to vehicle control from three independent experiments. * $p < 0.05$ relative to vehicle.

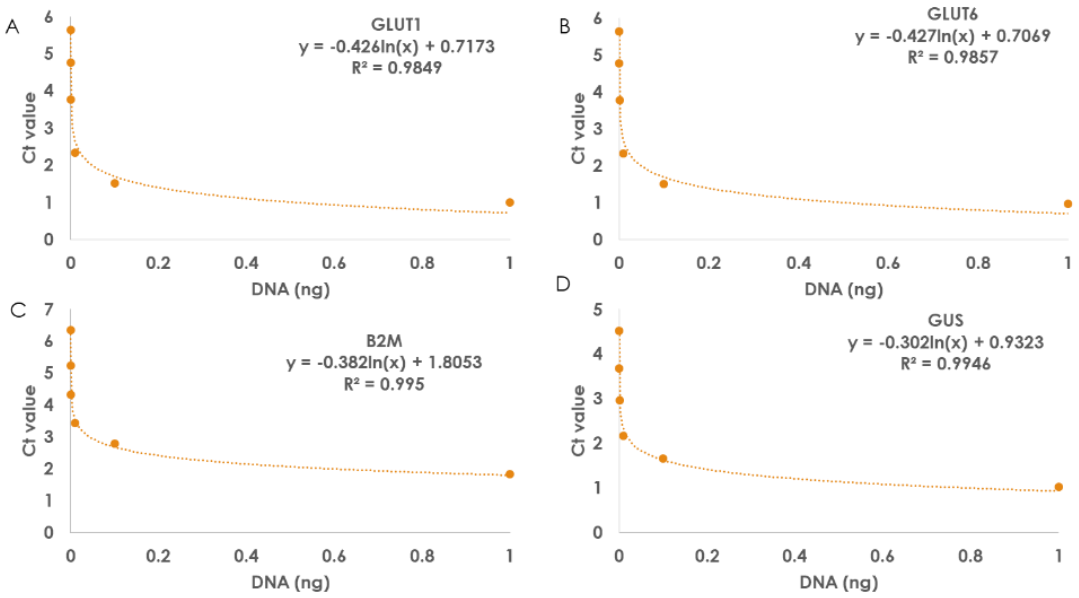

Figure 2. To directly quantify expression of each gene, standard curves of five 10-fold dilutions starting with 1 ng of A) GLUT1 B) GLUT6, and loading controls C) B2M and D) GUS DNA were generated for each experiment.

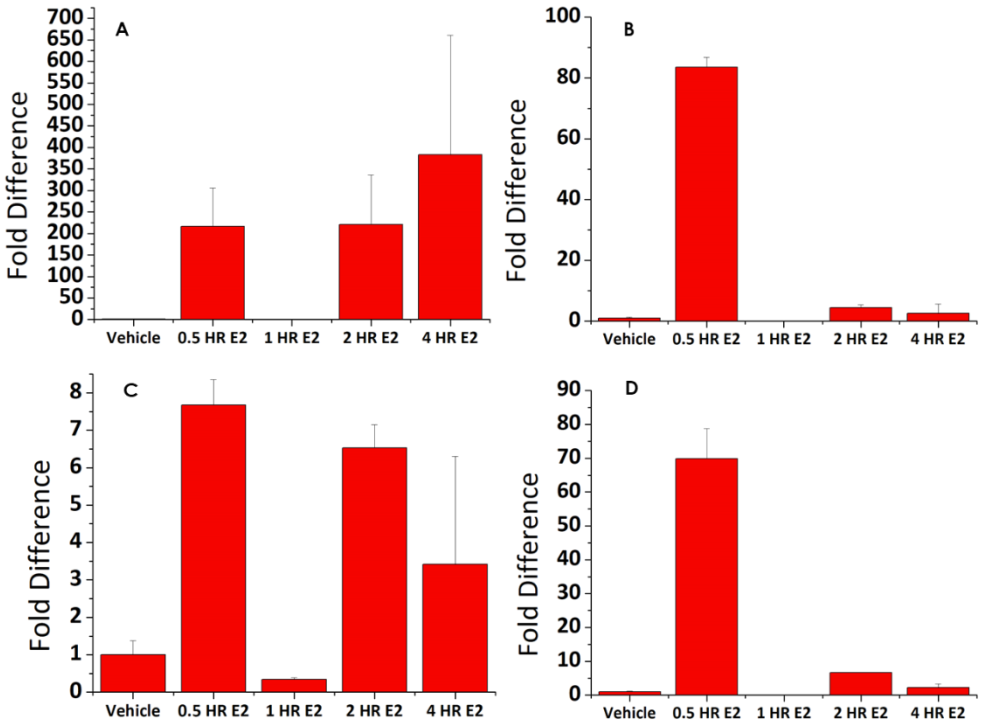

Figure 3. DNA amounts calculated from standard curves determined above (see Figure 2) for each of the indicated E2-stimulated time points for A) GLUT1, B) GLUT6, C) B2M, D) GUS. Amounts were normalized to an averaged value for the vehicle. Data indicate mean ± standard deviation of duplicates from a representative experiment.

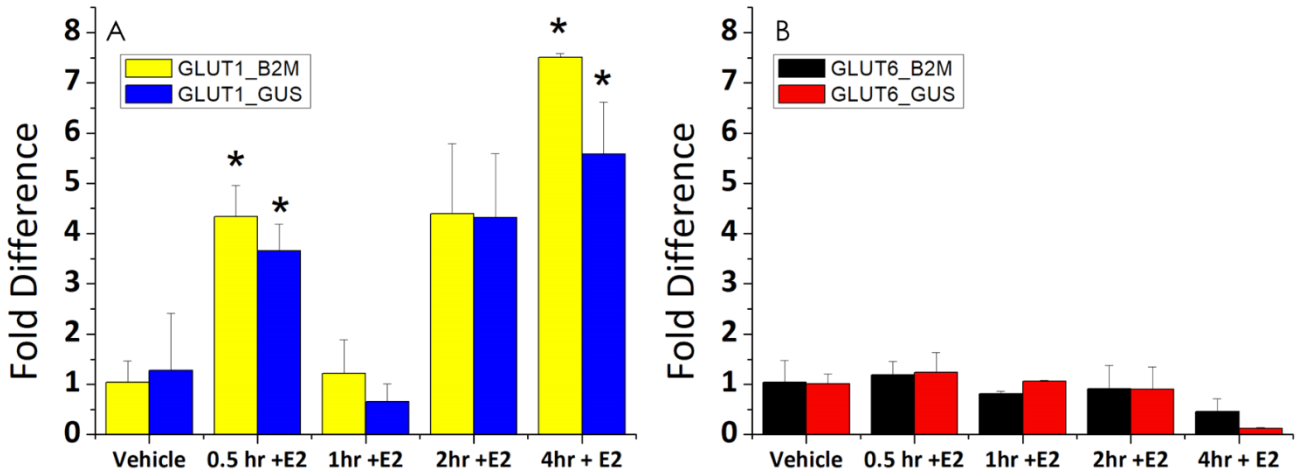

Figure 4. Fold difference in expression of A) GLUT1 and B) GLUT6 for each of the E2-stimulated time points using B2M and GUS as controls. Data indicate the mean of duplicates ± standard deviation of the $\Delta\Delta C_t$ values normalized to vehicle control. * $p < 0.05$ relative to vehicle control.

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