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Estradiol stimulates glycogen synthesis whereas progesterone promotes glycogen catabolism in the uterus of the American mink (*Neovison vison*)

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Abstract

Glycogen synthesis by mink uterine glandular and luminal epithelia (GE and LE) is stimulated by estradiol (E_2) during estrus. Subsequently, the glycogen deposits are mobilized to near completion to meet the energy requirements of pre-embryonic development and implantation by as yet undetermined mechanisms. We hypothesized that progesterone (P_4) was responsible for catabolism of uterine glycogen reserves as one of its actions to ensure reproductive success. Mink were treated with E_2 , P_4 or vehicle (controls) for three days and uteri collected 24h (E_2 , P_4 and vehicle) and 96h (E_2) later. To evaluate E_2 priming, mink were treated with E_2 for three days, then P_4 for an additional three days ($E_2 \rightarrow P_4$) and uteri collected 24h later. Percent glycogen content of uterine epithelia was greater at E_2+96h ($GE=5.71 \pm 0.55$; $LE=11.54 \pm 2.32$) than E_2+24h ($GE=3.63 \pm 0.71$; $LE=2.82 \pm 1.03$), and both were higher than controls ($GE=0.27 \pm 0.15$; $LE=0.54 \pm 0.30$; $P < 0.05$). Treatment as $E_2 \rightarrow P_4$ reduced glycogen content ($GE=0.61 \pm 0.16$; $LE=0.51 \pm 0.13$), to levels not different from controls, while concomitantly increasing catabolic enzyme (glycogen phosphorylase and glucose 6 phosphatase) gene expression and amount of phosphoglycogen synthase protein (inactive) in uterine homogenates. Interestingly, $E_2 \rightarrow P_4$ increased glycogen synthase 1 mRNA and hexokinase mRNA (and protein). Our findings suggest to us that while E_2 promotes glycogen accumulation by the mink uterus during estrus and pregnancy it is P_4 that induces uterine glycogen catabolism releasing the glucose that is essential to support pre-embryonic survival and implantation.

Keywords

Progesterone; estradiol; glycogen; uterus; mink

INTRODUCTION

Pre-embryonic growth, implantation and early fetal development are dependent upon uterine glandular and luminal epithelial cell secretions (histotroph) containing glucose, glycogen, amino acids, fats, ions and hormones (Gray *et al.* 2001; Burton *et al.* 2002; Hempstock *et al.* 2004). Although gluconeogenesis does not occur in the uterus (Zimmer & Magnuson 1990;

Yáñez *et al.* 2003) glucose is taken up, transported into the uterine lumen, metabolized as an energy substrate and when in excess, stored as glycogen.

Glycogen synthesis begins with phosphorylation of glucose by hexokinase (Hk), producing glucose-6-phosphate, which is isomerized to glucose-1-phosphate and converted to uridine diphosphate glucose (Ferrer *et al.* 2003). Glucosyl units from the latter are transferred to non-reducing ends of glycogen molecules by glycogen synthase (Gys). Glycogen is catabolized by glycogen phosphorylase (Pyg), releasing glucose-1-phosphate, which may be isomerized to glucose-6-phosphate and enter the glycolytic pathway, or be dephosphorylated by glucose-6-phosphatase (G6pc), releasing glucose for export to the systemic circulation and/or uterine lumen.

Uterine glycogen content in rodents peaks during proestrus to estrus, and is mobilized during implantation and pregnancy (Demers *et al.* 1972; Greenstreet & Fotherby 1973a). Similarly, the human uterus stores large amounts of glycogen during the late proliferative to early secretory phases, that is mobilized by the late secretory phase (Verma 1983; Cornillie *et al.* 1985; Spornitz 1992). In mink, uterine glandular and luminal epithelial glycogen content decreased 99% between estrus and the peri-implantation period (Dean *et al.* 2014). Mink are seasonal breeders exhibiting obligatory embryonic diapause and may have blastocysts (as many as 17) in a state of arrested development for 50–60 days *post coitum*, resulting in delayed implantation (Hansson 1947; Enders 1952). It is likely that mink uterine glycogen reserves are a potential source of energy that supports pre-embryonic survival and implantation. In support of this contention, the uterine glycogen content of sterile women or those exhibiting spontaneous abortions, was greatly diminished when compared to normal women and in some cases absent (Hughes *et al.* 1963,1969; Rubulis *et al.* 1965; Maeyama *et al.* 1977; Zawar *et al.* 2003; Girish *et al.* 2012).

Uterine glycogen synthesis is stimulated by estradiol (E₂) in rats, rabbits, guinea pigs (Demers *et al.* 1972; Demers *et al.* 1973a,b; Greenstreet & Fotherby 1973b) and mink (Rose *et al.* 2011). In the ovariectomized rat, exogenous E₂ increased uterine glycogen content as expected (Paul & Duttagupta 1973). However, when treated concomitantly with E₂ and progesterone (P₄), uterine glycogen concentrations were reduced when compared to E₂ alone. Demers and Jacobs (1973) treated ovariectomized rabbits and guinea pigs with E₂ followed by P₄ (E₂→P₄) to simulate E₂ priming and reported a reduction in uterine glycogen content and a 2-fold increase in glycogen phosphorylase activity, compared to E₂ alone. These findings suggest that P₄ promotes uterine glycogen catabolism and/or antagonizes the glycogenic actions of E₂.

Circulating E₂ concentrations in mink increase after mating, then decline during the variable period of embryonic diapause and increase again during the peri-implantation period (Lagerkvist *et al.* 1992). Plasma P₄ concentrations in mink remain low until the vernal equinox (regardless of the time of mating), then increase concomitantly with E₂ during the peri-implantation period. It is possible that E₂ promotes glycogen accumulation during proestrus and estrus, and that after mating and ovulation, P₄ induces glycogen mobilization. If this is true, the mink uterus must be extremely sensitive to P₄ at this time, since circulating concentrations of the steroid, while increasing, are very low until late diapause (Lagerkvist

et al. 1992). Nevertheless, increased uterine sensitivity to P₄, in response to E₂ induced up regulation of P₄ receptors, may result in a uterus that responds to low (perhaps unchanging) P₄ concentrations.

To determine if P₄ might induce glycogen catabolism and/or decrease glycogen synthesis in the mink uterus, animals were bilaterally ovariectomized and treated with E₂ and P₄ alone or to simulate E₂ priming, E₂ followed by P₄ (E₂→P₄). Uteri were analyzed for, (1). Glycogen content of endometrium, myometrium, stroma, glandular and luminal epithelia, (2). Relative mRNA expression levels for glycogen synthase 1 (*Gys1*), glycogen phosphorylase (*Pygm*), hexokinase 1 (*Hk1*), and glucose-6-phosphatase 3 (*G6pc3*), and (3). Protein levels for Gys1 and Hk1.

MATERIALS AND METHODS

Animals

Twenty-five adult (18 to 19 month old) dark preparturient female mink (6–8 offspring per litter), were moved from outdoor ranch conditions to the indoor animal facility at Idaho State University in early November. Mink were housed individually, fed a mixture of chicken and fish by-products daily and received water *ad libitum*. Animals were exposed to a daily photoperiod approximating natural photoperiodic changes for southeastern Idaho (Rose 1995), at room temperatures between 22–28°C. All procedures involving animal care, surgery and hormone treatments were approved by the Institutional Animal Care and Use Committee of Idaho State University (ISU), and conformed to the Guide for the Care and Use of Laboratory Animals (Protocol #6410909).

Hormones

Estradiol 17 beta (E₂; R187933; Sigma Chemical Co., St. Louis, MO, USA), and Progesterone (P₄; P0130; Sigma Chemical Co.) were initially dissolved in 95% EtOH and then mixed in sesame oil as a vehicle for SC injection.

Treatments

On Day 0 (Nov 17) all mink were bilaterally ovariectomized through a single mid-ventral incision while under ketamine hydrochloride anesthesia (50 mg/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA). Animals were subsequently returned to their cages and for 11 days, allowing for recovery and natural elimination of residual ovarian hormones. On Day 12, animals were assigned at random to one of five groups (Fig. 1; N = 5/group). Mink in groups 2, 3, and 4 were each injected once daily on days 12, 13 and 14 with E₂ (50 µg/kg body weight). Mink in group 5 each received daily injections of P₄ (25 mg/kg body weight) on days 12, 13 and 14 while mink in group 4 each received P₄ injections on days 15, 16 and 17. Animals in group 3 each received daily injections of vehicle on days 15, 16 and 17 while mink in group 1, received injections of vehicle only on days 12, 13 and 14 and represented controls. Twenty-four hours after the last perturbation, animals were killed with a lethal dose of Sleep-A-Way (Fort Dodge Animal Health), uteri were collected and quick frozen in liquid nitrogen.

Glycogen Concentrations in Uterine Homogenates

Glycogen concentrations were determined using a modified procedure of Good *et al.* (1933). Uterine tissue (70–120 mg) from each mink was lyophilized for 3 days and homogenized in 20 volumes of 30% KOH. A 125 μ L aliquot of the homogenate was incubated at 100°C for 30 min to denature enzymes and destroy free glucose. To isolate glycogen, samples were diluted with 1.2 vol (150 μ L) 95% EtOH, frozen at –80°C for 60 min, thawed and centrifuged at $9,600 \times g$ for 10 min. After discarding the supernatant, tubes were inverted and the pellet dried overnight. Subsequently, 100 μ L of 1.0 N HCL was added to the pellet, heated at 100°C for 2.5 h to break down glycogen to D-glucose. Glucose concentrations, as an indicator of glycogen content, were determined using Wako Glucose Auto Kit (439–90901; Wako Chemicals USA, Inc., Richmond, VA, USA) according to the manufacturer's instructions. Glucose was quantified spectrophotometrically ($\lambda = 505$ nm) by comparing unknowns against a standard curve of increasing glucose concentrations. All samples were analyzed in duplicate, in a single assay with an intra-assay coefficient of variation of 2.0%

Localization of glycogen deposits in uterine cross-sections

Uterine samples were fixed in 10% neutral buffered formalin, dehydrated and mounted in paraffin blocks. Transverse uterine sections (7 μ m) were incubated with Periodic Acid Schiff (PAS) reagent and counter stained with hematoxylin. Images were captured digitally at 25, 200 and 400 $\times$ and analyzed using ImageJ software (Abramoff *et al.* 2004). For quantification of endometrial and myometrial glycogen content, three consecutive uterine cross-sectional images from each mink were analyzed at 25 $\times$ (whole uterine cross section) whereas for glandular and luminal epithelial glycogen content, a portion (at 400 $\times$) of each of three consecutive uterine cross sections were analyzed. Because PAS stains carbohydrates in addition to glycogen, an additional cross-section was pre-treated with diastase (A8220; Sigma Chemical Co.), to digest glycogen prior to PAS staining and served as a negative control. Glycogen content of each tissue was quantified by subtracting negative control values from sections stained with PAS without diastase and then expressed as a percentage of the area that stained positive for glycogen.

RNA isolation and conversion to cDNA

Total RNA was isolated from 20–30 mg uterine tissue from each mink using Qiagen RNeasy Fibrous Tissue Mini Kit (74104; QIAGEN, Valencia, CA, USA) as previously described (Rose *et al.* 2011). Samples were screened for protein contamination by measuring light absorption of each sample at 260 nm (DNA and RNA) and 280 nm (protein). Only RNA preparations with 260/280 ratio of 1.9 or greater were used for qPCR analysis. To eliminate any contaminating DNA, samples were treated with genomic (gDNA) wipeout buffer prior to the reverse-transcription reaction. Conversion of unstable RNA to stable cDNA transcripts (first strand transcripts) was achieved using QuantiTect Reverse Transcription Kit (205311; QIAGEN) according to the manufactures instruction.

Primer design

To produce primers specific to mink cDNA, we first used the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI; NIH <http://>

www.ncbi.nlm.nih.gov/sites/entrez?dbgenome), and designed conventional PCR primers for each gene of interest based on regions of homology between rats and other species. Using these primers, we amplified mink cDNA using the Qiagen HotStarTaq® Master Mix Kit (203443; QIAGEN). We separated the resulting cDNA fragments by gel-electrophoresis and the band containing the gene fragment of interest were purified using a Millipore Ultra Free DNA Centrifugal Filter (42600; Millipore Corporation, Billerica, MA, USA). The nucleotide sequence for each gene of interest was then determined using Biosystems 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Based on the resulting mink nucleotide sequence for each target gene of interest, primers were designed for qPCR (Table 1), and purchased from Integrated DNA Technologies (Coralville, IA, USA).

Quantitative PCR (qPCR)

All qPCR reactions were carried out in triplicate using QuantiTect SYBR® Green PCR Kit (204143; QIAGEN) as per the manufacture's instructions. Forward and reverse primer concentrations were previously optimized and were added to 5 µl cDNA template (100–200 ng) per reaction. The PCR products were detected in real time by measuring SYBR-green fluorescence during the annealing stage using MJ Research Chromo4 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Efficiency of amplicon doubling during each PCR cycle was, *Gapdh*: 97%, *Gys1*: 103%, *Pygm*: 98%, *G6pc3*: 97%, and *Hk1*: 100%. Negative controls contained no template and amplification was never above background. Primer specificity was determined using melt curve analysis, which resulted in a single melting temperature for each amplicon.

Fold changes in gene expression were determined using the relative standard curve method (Pfaffl 2001). Standard curves were generated from 3 pooled control mink uteri. The cDNA from these uteri was diluted (1:10, 1:100, 1:1,000 and 1:10,000) or undiluted to construct standard curves (cDNA ng/mL versus quantification cycle, Cq) for each gene of interest ($R^2 = 0.98–0.99$). Data were averaged by treatment group ($n = 5$ mink/group, each assayed in triplicate) and expressed in terms of relative fold-difference compared to controls.

We chose *Gapdh* with which to normalize our data, as it showed the least variation in expression (*Gapdh* Cq for E₂+24h = 21.59 ± 0.22 , for E₂+96h, Cq = 21.54 ± 0.38 , for E₂→P₄, Cq = 21.64 ± 0.55 , for P₄ +24 h = 21.72 ± 0.33 and for controls, Cq = 22.28 ± 0.22), as compared to *18s rRNA* (*18s rRNA* Cq for E₂+24h, = 17.47 ± 0.58 , for E₂+96h, Cq = 18.34 ± 0.39 , for E₂→P₄, Cq = 17.97 ± 0.07 , for P₄ +24 h = 18.60 ± 0.56 and for Controls, Cq = 23.05 ± 0.48).

Western blot analysis

Uterine proteins were isolated using RIPA Lysis Buffer (sc-24948 Santa Cruz Biotechnology, Dallas, TX, USA) according to the manufacturer's instructions. Protein concentrations were determined using Pierce BCA Protein Assay Kit (23225; Thermo Scientific, Rockford, IL, USA). Enzyme proteins of interest were resolved by molecular weight using SDS-PAGE, transferred to nitrocellulose membranes, and blocked for 1h in 5% milk buffer containing TBS-Tween 20 (BP337-100; Invitrogen, Pittsburgh, PA, USA) to reduce non-specific binding. Membranes were subsequently incubated overnight at 4°C with

primary antibodies specific for Gys (3886S; Cell Signaling Technology, Danvers, MA, USA, 1:500 dilution), phosphorylated Gys (pGys: 3891S; Cell Signaling Technology, Danvers, MA, USA, 1: 500 dilution), Hk1 (2024S; Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution). Subsequently the membranes were washed in TBS-T to remove excess primary antibodies. At that time we incubated the membranes with secondary antibody (anti rabbit IgG conjugated to horseradish peroxidase; 7074, Cell Signaling Technology, 1:1000 dilution) for 2 h to detect the enzyme proteins of interest. Concomitantly (as a loading control) we incubated membranes with a primary antibody conjugated to horseradish peroxidase against beta-actin (Actb-HRP, 5125S; Cell Signaling Technology, 1:10,000 dilution) for 2 h. To blots with HRP conjugated to the secondary antibody we added Immobilon Western Chemiluminescent HRP Substrate (WBKLS0100; Millipore Corporation), while samples that were incubated with Actb-HRP were incubated with Novex HRP Chromogenic Substrate (100002903; Invitrogen, Grand Island, NY, USA). The resulting blots were photographed using Bio-Rad VersaDoc 3000 Imager (Bio-Rad Laboratories). The relative amount of each protein of interest was determined using ImageJ gel analyzer to quantify band density and then normalized to beta actin as a loading control. Data was then expressed as number of pixels per unit area, with all samples for each mink measured in triplicate.

To validate the use of primary antibodies against mink proteins, both mink and rat proteins were analyzed concurrently. For each protein assayed we detected only a single band corresponding to the correct molecular weight of each protein (Gys, pGys and Hk1) in both rat and mink uterine tissue (data not shown). All primary antibodies were produced against human proteins and validated by the manufacturer against rat proteins. Antibodies against Gys were not specific to a single isoform and would therefore bind to both Gys1 (muscle) and Gys2 (liver).

Statistical Analysis

All data were analyzed by one-way ANOVA, followed by Tukey's post- hoc test (SigmaPlot, version 12.5; Systat Software Inc., San Jose, CA, USA). Differences were considered significant at $P = 0.05$.

RESULTS

Uterine endometrial area was 4-fold greater at 24h and 6-fold greater at 96h after the last E_2 injection when compared to ovariectomized controls (Table 2; $P < 0.05$). The endometrial area of mink treated as $E_2 \rightarrow P_4$, was less than E_2+96h treated animals ($P < 0.05$), but did not differ from E_2+24h mink. Although the endometrial area of $E_2 \rightarrow P_4$ and P_4+24h treated mink tended to be greater than controls, the differences were not significant. Myometrial area was greater at 24 h (3.5 fold) and 96 h (2.5 fold) after the last E_2 injection when compared to controls (Table 2; $P < 0.05$). The myometrial area of mink treated as $E_2 \rightarrow P_4$, did not differ from mink treated with E_2 only (24 and 96h), and was greater when compared to controls ($P < 0.05$). Myometrial area of mink treated with P_4+24h did not differ from ovariectomized controls.

Gross uterine glycogen concentrations in mink treated as E₂+24h were higher than for all other treatments (Table 3, *P* 0.05). In E₂+96h treated mink, uterine glycogen concentrations were higher than controls (*P* 0.05), but approximately 50% less than for E₂+24h treated mink (*P* 0.05). For E₂→P₄ treated mink uterine glycogen content was 70% less than for E₂+24h treated animals (*P* 0.05) and 40% less than mink treated as E₂+96h. Treatment with P₄+24h had no affect on gross uterine glycogen content when compared to controls.

ImageJ analyses revealed that both endometrial and myometrial glycogen content were higher in E₂+24h and E₂+96h treated mink, when compared to all other treatments (Table 3, *P* 0.05). The glycogen content of both tissues tended to be lower in E₂+96h mink when compared to E₂+24h animals but the differences were not significant. Mink treated as E₂→P₄ had endometrial and myometrial glycogen concentrations that did not differ from controls or mink treated as P₄+24h.

Glycogen content of the glandular and luminal epithelia was highest in E₂+96h mink when compared to all other treatments (Table 4; *P* 0.05). In E₂+24h mink, glycogen content of both epithelia was less than for E₂+96h mink, but higher than mink treated with P₄+24h, E₂→P₄ or as controls (*P* 0.05). There was no difference in glycogen content of glandular and luminal epithelia among mink treated as E₂→P₄, P₄+24h or controls. Stromal cell glycogen content was highest in E₂+96h treated mink when compared to all other treatments (*P* 0.05). There was no difference in stromal cell glycogen content among mink treated as E₂→P₄, E₂+24h or controls. Stromal glycogen content was lowest in response to P₄+24h when compared to all other groups (*P* 0.05).

Uterine hexokinase 1 mRNA expression in E₂+24h mink was 30% greater than controls (*P* 0.05; Fig. 3). There was no difference in expression of the gene in E₂+96h mink or in response to P₄+24h, when compared to controls. When mink were primed with E₂ and subsequently treated with P₄ (E₂→P₄) *Hk1* mRNA expression was higher than for all other treatments (*P* 0.05).

Uterine glycogen synthase 1 mRNA expression was greatest in response to E₂→P₄ when compared to all other treatments, followed by E₂+96h and P₄+24h (Fig. 3; *P* 0.05). Mink treated as E₂+24h exhibited Glycogen synthase 1 mRNA expression levels that did not differ from controls.

Expression of glycogen phosphorylase m mRNA by whole uterine homogenates for E₂+24h and E₂+96h treated mink did not differ from controls (Fig. 3). Mink primed with E₂ and then treated with P₄ (E₂→P₄) or P₄+24h had higher glycogen phosphorylase m gene expression levels when compared to mink treated as E₂+24h, E₂+96h or as controls (*P* 0.05).

Uterine glucose 6 phosphatase 3 mRNA expression was greater for E₂+96h treated mink when compared to E₂+24h or controls (Fig. 3; *P* 0.05). Mink treated as E₂→P₄ showed the highest level of glucose 6-phosphatase 3 gene expression, followed by treatment with P₄+24h.

Hexokinase 1 and glycogen synthase proteins in whole uterine homogenates for mink treated as E₂+24h and E₂+96h or in response to P₄+24h did not differ from controls (Fig.

4). The amount of both proteins in response to $E_2 \rightarrow P_4$ was greater when compared to all other treatments ($P = 0.001$). Similarly, the amount of phospho-glycogen synthase protein was greater in response to $E_2 \rightarrow P_4$ when compared to all other treatments ($P = 0.05$). Mink treated as $E_2 + 24h$ had lower phospho-glycogen synthase protein content when compared to $E_2 \rightarrow P_4$ or $E_2 + 96h$ treatments ($P = 0.05$), and tended to be lower than mink treated with $P_4 + 24h$.

DISCUSSION

Glycogen content of the mink uterine myometrium, endometrium, stroma, luminal and glandular epithelia were increased by exogenous E_2 at 24 and 96 h after the last injection when compared to controls ($P = 0.05$; Tables 3 and 4, Fig. 2). These findings support our previous observations for mink (Rose *et al.* 2011) and agree with those for rats, rabbits and guinea pigs (Bitman *et al.* 1965; Bitman & Cecil 1967; Gregoire *et al.* 1967; Hall & Khaligh 1968; Demers *et al.* 1973a; Tripathi 1983).

Because circulating E_2 concentrations in mink are increasing for weeks prior to proestrus and estrus, (Pilbeam *et al.* 1979), it would appear that part of the effects of E_2 on the mink uterus are to promote glycogen accumulation in preparation for the breeding season. Our recent findings of peak uterine glycogen content in mink during estrus, that diminished throughout implantation and pregnancy (Dean *et al.* 2014) supports this hypothesis. It would not seem unreasonable to propose that without adequate uterine glycogen reserves, and their subsequent mobilization, mink pre-embryos may fail to survive and/or develop to the blastocyst stage. This could have a very negative impact on resulting litter sizes.

The glycogen content of whole uteri of $E_2 + 96h$ treated mink was less than for $E_2 + 24h$ treated animals ($P = 0.05$; Table 3). Similarly, the glycogen content of the endometrium and myometrium exhibited the same trend, although the differences were not significant. Such findings might suggest that glycogen catabolism predominated over glycogen synthesis between hours 24 and 96, and that elevated E_2 concentrations must be maintained above some critical level to maintain a higher glycogen synthesis to catabolism ratio. And yet, the glycogen content of the glandular epithelia, luminal epithelia and stroma were greater in mink treated as $E_2 + 96h$ than $E_2 + 24h$ ($P = 0.05$; Table 4). We reasoned that glycogen determinations made by ImageJ analysis of the endometrium, myometrium, stroma and whole uterine glycogen measurements were low at 96 h after the last E_2 injection, as a result of being diluted by the relatively large amount of extracellular material and cells not containing glycogen (Fig. 2). As mentioned previously, the endometrial area was approximately 4-fold greater at 24h and 6-fold greater at 96 h after the last E_2 injection (Table 2).

Even after using ImageJ to isolate the stromal compartment of the endometrium, the glycogen content for the stroma was orders of magnitude below that detected for the glandular and luminal epithelia. Because of the small amount of glycogen detected within such a large area, we believe that our glycogen content measurements for the stroma may approach the limits of detection by ImageJ analysis and may not be an accurate representation of glycogen content of the stromal cells. By contrast, the epithelia can be very

precisely delineated with ImageJ. As a result we believe that glycogen content determinations for glandular and luminal epithelia are the most accurate, and physiologically meaningful measurements, since the epithelia is the immediate source of uterine histotroph.

The glycogen content of the glandular epithelia, luminal epithelia and stroma was significantly greater in E₂+96h than E₂+24h treated animals (Table 4). This illustrates that E₂ had prolonged effects on glycogen synthesis in these cells. In agreement with this finding, glycogen synthase 1 mRNA levels in uterine homogenates were higher in E₂+96h mink when compared to E₂+24h or controls (Fig. 3; $P = 0.05$). Although we could detect no difference in glycogen synthase 1 protein by WBA among E₂+24h, E₂+96h and controls (Fig. 4), the amount of phospho-glycogen synthase protein (inactive) was significantly lower in E₂+24h mink, when compared to controls, which could have contributed to increased glycogen synthesis.

Exogenous E₂ had no effect on glycogen phosphorylase m gene expression at 24 or 96 h after the last injection (Fig. 3). This agrees with the general consensus that E₂ is glycogenic in the uterus. However, when mink were treated as E₂→P₄, or P₄+24h, uterine glycogen phosphorylase m gene expression was higher than E₂+24h, E₂+96h or controls (Fig. 3; $P = 0.05$). Moreover, phospho-glycogen synthase protein and glucose 6 phosphatase 3 mRNA were also highest in response to E₂→P₄ treatment (Figs 3 and 4). In agreement with this observation we previously showed that expression of glucose 6 phosphatase 3 mRNA was five-fold greater during late diapause (as P₄ levels are increasing) and three-and-a-half-fold greater during pregnancy when compared to estrus (Dean *et al.* 2014). Collectively, these findings suggest that P₄ promotes glycogen catabolism in the mink uterus.

It is well known that glycogen synthesis and catabolism occur concomitantly within cells and that net glycogen content reflects the relative levels of anabolic and catabolic processes. Thus, even though glycogen reserves in the mink uterus are almost depleted prior to implantation, the level of anabolic activity could still be very high at this time and play a significant role in sequestering maternal glucose to the uterus. In agreement with this supposition, we show that hexokinase 1 mRNA and protein were higher in mink in response to E₂→P₄ when compared to all other treatments (Figs 3 and 4). Recently, we showed that hexokinase 1 protein, detected by immunohistochemistry in mink uterine epithelia was high during estrus and peri-implantation (Dean *et al.* 2014). It is possible that increased hexokinase expression in response to P₄ contributes to increased trapping of glucose, from the maternal circulation; even after uterine glycogen reserves are depleted. Similarly, increased glycogen synthase expression in response to E₂→P₄, (even if catabolism predominated over anabolism) could serve to divert maternal glucose to the uterus. It was therefore, not surprising to discover that some glycogenic processes in the uterus were enhanced in E₂→P₄ treated mink.

In summary, we conclude that mink pre-embryonic survival and development to the blastocyst stage depends in part, upon P₄ induced uterine glycogen catabolism and release of glucose. During implantation, as circulating E₂ and P₄ concentrations are increasing, P₄ actions may predominate over E₂, resulting in a greater level of glycogen catabolism than synthesis. Nevertheless, E₂ independently or in combination with P₄ may still promote

glycogen synthesis at this time. This latter affect could promote the continued sequestration of glucose from the maternal circulation to the uterus ensuring adequate nutritional support for embryo implantation and early pregnancy. Moreover, we believe that circulating P₄ concentrations must be above some critical level, between mating and implantation, to ensure reproductive success, and maximize litter sizes. As evidence we recently showed that fecal P₄ concentrations from mated female mink increased earlier and were significantly greater in all animals that whelped when compared to those that failed to whelp (Cao *et al.* 2015). We propose that reproductive failures in mink may in part be due to (1): an insufficient quantity of glycogen being accumulated by the uterus during pro-estrus and estrus, (2): inadequate mobilization of uterine glycogen reserves during pre-embryonic development and implantation, and/or (3): failure to divert sufficient amounts of glucose from the maternal circulation to the uterus during early pregnancy. In addition to the many documented actions of P₄ on the uterus, it would now seem that regulation of uterine glycogen metabolism by the hormone should attract the attention of investigators as a potential target for increasing reproductive performance as well as the development of newer and perhaps safer means of birth control.

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Figure 1.

Twenty-five adult female mink were bilaterally ovariectomized on Nov 17 (Day 0). On Day 12, mink were assigned at random to one of 5 groups ($N = 5/\text{group}$) and treatments initiated. Mink in groups 2, 3 and 4 were each injected once daily, on days 12, 13 and 14 with estradiol 17beta (E_2 ; 50 $\mu\text{g}/\text{kg}$ body weight). On the same days, mink in group 5 each received daily injections of progesterone (P_4 ; 25 mg/kg body weight). Mink in group 3, received daily injections of vehicle on days 15, 16 and 17 while mink in group 4 received daily injections of P_4 on the same days to simulate E_2 priming. Mink in group 1 received injections of vehicle only on days 12, 13 and 14 and represented controls. All animals were sacrificed 24h after the last perturbation (▼) with a lethal dose of Sleep-Away (Fort Dodge Animal Health, Fort Dodge, IA, USA), the uteri collected and quick frozen in liquid nitrogen.

Figure 2.

Mink uterine cross-sectional images. Animals were injected with E₂ for three days and sacrificed 24h (E₂+24h) and 96h (E₂+96h) after the last injection. To simulate E₂ priming, mink were treated for three days with E₂ and then an additional three days with P₄ and sacrificed 24h later (E₂→P₄). Mink were also treated with P₄ for three days and sacrificed 24h later (P₄+24h). Controls (CON) received injections of vehicle only for three days and were sacrificed 24h later. All sections (7 μm) were stained with Periodic Acid Schiff (PAS) and counterstained with hematoxylin. Images were captured at 25 × (left column), 200 × (middle column) and 400 × (right column). Arrows identify areas of positive PAS staining

within the glandular lumens. M, myometrium; E, endometrium, GE, glandular epithelium; LE, luminal epithelium

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Figure 3.

Relative (mean $\pm$ SE) mRNA expression levels for hexokinase 1, glycogen synthase 1, glycogen phosphorylase m and glucose 6-phosphatase in mink uterine homogenates. Animals were injected with E₂ for three days and sacrificed 24h (E₂+24) and 96h (E₂+96) after the last injection. To simulate E₂ priming, mink were treated for three days with E₂ and then an additional three days with P₄ and sacrificed 24h later (E₂→P₄). Mink were also treated with P₄ for three days and sacrificed 24h later (P₄+24). Controls (CON) received injections of vehicle only for three days and were sacrificed 24h later. Expression levels represent the mean of five mink, each analyzed in triplicate. Groups without a common letter differ at P < 0.05.

Figure 4.

Western blot analyses for hexokinase 1, phosphoglycogen synthase 1 and glycogen synthase proteins in mink uterine homogenates. Animals were injected with E₂ for three days and sacrificed 24h (E₂+24) and 96h (E₂+96) after the last injection. To simulate E₂ priming, mink were treated for three days with E₂ and then an additional three days with P₄ and sacrificed 24h later (E₂→P₄). Mink were also treated with P₄ for three days and sacrificed 24h later (P₄+24). Controls (CON) received injections of vehicle only for three days and

were sacrificed 24h later. Expression levels represent the mean of five mink, each analyzed in triplicate. Groups without a common letter differ at $P = 0.05$.

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Table 1

Primers for qPCR of mink uterine gene transcripts and amplicon characteristics

Genes	Forward and reverse primers (5' to 3')	Amplicon (bp)	Melt temperature (°C)	Accession No.
<i>Gys1</i>	AAGATGGCAGCCACGGACAAATTC TCAATAGTAGTGCCGACAGGGTCA	110	87.5 ± 0.50	KM084858
<i>Pygm</i>	TCATCACCCCTGTACAACCGCATCA AGCCTTCCCTCCAATCATCACAGT	80	79.0 ± 0.35	KM084856
<i>Hk1</i>	AATGCCAAAGAAATCCTGACCCGC TGGGTGTGCCCTTGTATCTCGAA	163	84.1 ± 0.43	KM026508
<i>G6pc3</i>	CCCTACTGCCTCGCCCAGGT TGGGCTTGTGAGCTGGCCCTA	117	86.4 ± 0.50	KM015512
<i>Gapdh</i>	AGGTCATCCATGACAACCTTCGGCA ACCAGTGGAAGCAGGGATGATGTT	152	84.8 ± 0.45	KM025344

Gys1, glycogen synthase 1; *Pygm*, glycogen phosphorylase-muscle; *Hk1*, hexokinase 1; *G6pc3*, glucose 6-phosphatase 3; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

Table 2

Mean ($\pm$ SE) uterine endometrial and myometrial areas (pixels $\times 10^4$). Mink in Groups 2, 3 and 4 were each injected once daily on days 12, 13 and 14 with estradiol 17beta (E_2 ; 50 μ g/kg body weight). On the same days, mink in Group 5 each received daily injections of progesterone (P_4 ; 25mg/kg body weight). Mink in Group 3 received vehicle only on days 15, 16 and 17 while mink in Group 4 received daily injections of P_4 on the same days. Mink in group 1 received vehicle only on days 12, 13 and 14 and represented controls. All mink were sacrificed approximately 24h after the last perturbation

Group	Treatment	Endometrial area, (pixels $\times 10^4$)	Myometrial area (pixels $\times 10^4$)
1	Control	3.87 $\pm$ 0.65 ^a	7.13 $\pm$ 0.57 ^a
2	E_2 + 24h	16.14 $\pm$ 1.83 ^b	24.96 $\pm$ 4.00 ^b
3	E_2 + 96h	24.87 $\pm$ 3.64 ^c	19.58 $\pm$ 1.20 ^{bc}
4	$E_2 \rightarrow P_4$	11.55 $\pm$ 1.37 ^{ab}	20.31 $\pm$ 4.14 ^{bc}
5	P_4 + 24h	8.59 $\pm$ 0.45 ^{ab}	9.67 $\pm$ 0.54 ^{ac}

^{a-c} Within a column, means without a common superscript differ ($P < 0.05$)

Table 3

Mean ($\pm$ SE) glycogen concentrations (mg/g dry weight) in whole uteri, and percent glycogen content of endometrium and myometrium (% area PAS positive $\times$ 10). Mink in groups 2, 3, and 4 were each injected once daily, on days 12, 13 and 14 with estradiol 17beta (E_2 ; 50 μ g/kg body weight). On the same days, mink in group 5 each received daily injections of progesterone (P_4 ; 25 mg/kg body weight). Mink in group 3 received vehicle only on days 15, 16 and 17 while mink in group 4 received daily injections of P_4 on the same days. Mink in group 1 received vehicle only on days 12, 13 and 14 and represented controls. All mink were sacrificed approximately 24h after the last perturbation. Relative fold-increase above controls are given in parentheses

Group	Treatment	Glycogen (mg/g dry weight)	Endometrial glycogen (% Area PAS+)	Myometrial glycogen (% Area PAS+)
1	Control	2.72 ± 0.29^a	0.62 ± 0.39^a	2.79 ± 1.57^a
2	$E_2 + 24h$	21.91 ± 3.09^b (8)	25.00 ± 4.77^b (40)	25.50 ± 4.82^b (9)
3	$E_2 + 96h$	11.27 ± 2.04^c (4)	17.39 ± 2.75^b (28)	15.70 ± 3.67^b (5)
4	$E_2 \rightarrow P_4$	6.80 ± 0.44^c (2)	4.39 ± 1.33^a (7)	1.69 ± 0.31^a
5	$P_4 + 24h$	3.79 ± 0.54^a	0.02 ± 0.01^a	1.68 ± 0.57^a

^{a-c} Within a column, means without a common superscript differ ($P \leq 0.05$).

Table 4

Mean ($\pm$ SE) percent glycogen content of uterine glandular and luminal epithelia and stroma (% area PAS positive $\times$ 10). Mink in groups 2, 3 and 4 were each injected once daily, on days 12, 13 and 14 with estradiol 17beta (E_2 ; 50 μ g/kg body weight). On the same days, mink in group 5 each received daily injections of progesterone (P_4 ; 25 mg/kg body weight). Mink in group 3 received vehicle only on days 15, 16, and 17 while mink in group 4 received daily injections of P_4 on the same days. Mink in group 1 received vehicle only and represented controls. All animals were sacrificed approximately 24h after the last perturbation. Relative fold-increase above controls are given in parentheses.

Group	Treatment	Glandular epithelia	Luminal epithelia	Stroma
1	Control	0.27 ± 0.15^a	0.54 ± 0.30^a	0.089 ± 0.014^a
2	$E_2 + 24h$	3.63 ± 0.71^b (13)	2.82 ± 1.03^b (5)	0.055 ± 0.013^a
3	$E_2 + 96h$	5.71 ± 0.55^c (20)	11.54 ± 2.32^c (21)	0.141 ± 0.036^b
4	$E_2 \rightarrow P_4$	0.61 ± 0.16^a (2)	0.51 ± 0.13^a	0.054 ± 0.018^a
5	$P_4 + 24h$	0.01 ± 0.01^a	0.14 ± 0.05^a	0.013 ± 0.008^c

^{a-c} Within a column, means without a common superscript differ ($P < 0.05$)