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The effects of estradiol and catecholestrogens on uterine glycogen metabolism in mink (*Neovison vison*)

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Abstract

Glycogen is a uterine histotroph nutrient synthesized by endometrial glands in response to estradiol. The effects of estradiol may be mediated, in part, through the catecholestrogens, 2-hydroxycatecholestradiol (2-OHE2) and 4-hydroxycatecholestradiol (4-OHE2), produced by hydroxylation of estradiol within the endometrium. Using ovariectomized mink, our objectives were to determine the effects of estradiol, 4-OHE2, and 2-OHE2 on uterine: 1) glycogen concentrations and tissue localization; 2) gene expression levels for glycogen synthase, glycogen phosphorylase, and glycogen synthase kinase-3B; and 3) protein expression levels for glycogen synthase kinase-3B (active) and phospho-glycogen synthase kinase-3B (inactive). Whole uterine glycogen concentrations (mean $\pm$ SEM, mg/g dry wt) were increased by estradiol (43.79 ± 5.35), 4-OHE2 (48.64 ± 4.02), and 2-OHE2 (41.36 ± 3.23) compared to controls (4.58 ± 1.16 ; $P \leq 0.05$). Percent glycogen content of the glandular epithelia was three-fold greater than the luminal epithelia in response to estradiol and 4-OHE2 ($P \leq 0.05$). Expression of glycogen synthase mRNA, the rate limiting enzyme in glycogen synthesis, was increased by 4-OHE2 and 2-OHE2 ($P \leq 0.05$), but interestingly, was unaffected by estradiol. Expression of glycogen phosphorylase and glycogen synthase kinase-3B mRNAs were reduced by estradiol, 2-OHE2, and 4-OHE2 ($P \leq 0.05$). Uterine phospho-glycogen synthase kinase-3B protein was barely detectable in control mink, whereas all three steroids increased phosphorylation and inactivation of the enzyme ($P \leq 0.05$). We concluded that the effects of estradiol on uterine glycogen metabolism were mediated in part through catecholestrogens; perhaps the combined actions of these hormones are required for optimal uterine glycogen synthesis in mink.

Keywords

Uterus; Glycogen; Glycogen synthase (Gys); Glycogen synthase kinase-3B (Gsk3B); Glycogen phosphorylase (Pyg); Catecholesterogen; Estradiol

1. Introduction

Mink exhibit obligatory embryonic diapause and may have blastocysts up to 60 d of age (*post coitum*) at implantation, giving birth to as many as 17 offspring [1,2]. Until formation of the placenta is complete, embryonic growth and development depend on uterine glandular

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secretions or histotroph, containing enzymes, hormones, growth factors, and nutrients [3,4]. Uterine histotroph is rich in a variety of carbohydrates, including glycogen [5–7]. In anestrous mink, uterine glycogen deposits were detected in luminal but not glandular epithelia [8]. During estrus and embryonic diapause, glycogen deposits were detected in luminal and glandular epithelium, and decreased after implantation [9–11]. The post-implantation reduction in uterine glycogen content, which has also been reported in cats, [12] armadillos [13], and ferrets [14], is thought to reflect utilization of the nutrient by embryos.

Glycogen synthesis is catalyzed by glycogen synthase, whereas catabolism is controlled by glycogen phosphorylase [15]. The enzyme glycogen synthase kinase-3B, which is constitutively active in uterine tissue [16,17], phosphorylates glycogen synthase, reducing its activity and glycogen synthesis. Similarly, phosphorylation and inactivation of glycogen synthase kinase-3B reduces inhibition on glycogen synthase, leading to increased glycogen synthesis. Glycogen synthesis in the uteri of rats, rabbits, and guinea pigs is increased by estradiol [18–21]. Moreover, the effects of estradiol may be mediated in part, through the catecholestrogens, 2-hydroxycatecholestradiol (2-OHE2) and 4-hydroxycatecholestradiol (4-OHE2), following hydroxylation of the parent hormone by the uterine endometrium [22–24]. In the mouse, 4-OHE2 activated dormant blastocysts [25] and upregulated expression of the lactoferrin gene in the uterus [26]. Catecholestrogens bound to conventional receptors for estradiol, but may also act through distinctly separate signaling pathways [23,26]. Due to their rapid metabolic clearance, it is unlikely that catecholestrogens function as circulating hormones, but rather act as autocrine, paracrine, and intracrine mediators of the effects of estradiol [23,27–29]. In the present study, we tested the hypothesis that the effects of estradiol on uterine glycogen metabolism in the mink may be mediated in part through catecholestrogens.

2. Materials and methods

2.1. Animals and treatments

Twenty-four adult (15 to 16 mo old) female mink were moved to the indoor animal facility on the Idaho State University (ISU) campus during late August. All mink were primiparous, high producers, having given birth to a litter of 6 to 8 offspring the previous spring. Animals were housed individually, fed a mixture of chicken and fish by-products daily, received water *ad libitum*, and exposed to a photoperiod approximating natural changes in day length for Southeastern Idaho, with an Electronic Astronomic Time Switch, Model ET816CR (Intermatic Corp., Spring Grove, IL, USA). Mink were illuminated with General Electric Full Spectrum Chroma-50, Model F40C50 lamps, and maintained at a room temperature of 25 ± 3 °C. Animal care and research procedures were approved by the Institutional Animal Care and Use Committee of ISU, and complied with the Guide for the Care and Use of Laboratory Animals. Between September 6 and 7 (Day 0), 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, Ft. Dodge, IA, USA), and returned to their cages to recover and allow natural elimination of residual ovarian hormones. Subsequently, mink (N=6/group) were injected twice daily (0600 and 1400) on Days 12, 13, and 14 (Sept 18, 19, 20) with 200 µg/kg body weight of estradiol, 4-OHE2, or 2-OHE2 (R187933, H4637, H3131 respectively; Sigma Chemical Co., St. Louis MO, USA), in sesame seed oil, whereas control mink received oil injections only. On Day 15, each mink was anesthetized with ketamine hydrochloride, the uterus removed, weighed, and quick frozen in liquid nitrogen. All animals were then killed with a lethal dose of Sleep-A-Way (Fort Dodge Animal Health).

2.2. Glycogen determination for uterine homogenates

Uterine glycogen concentrations were determined according to Passonneau and Lauderdale [30]. A uterine sample (50 mg) from each animal was lyophilized, and homogenized in 0.03 M HCl (Sample A). To a 50 μ L aliquot of Sample A, 200 μ L of 1.0 M HCl was added, followed by incubation at 100° C for 4 h (Sample B), to break glycogen down to glucose. Glucose was detected using infinity glucose hexokinase reagent (TR15498; Fisher Scientific, Pittsburgh, PA, USA) and quantified spectrophotometrically (λ = 340 nm), by comparing unknowns against a standard curve of increasing glucose concentrations. Total glycogen concentrations were determined by subtracting the free glucose concentration of Sample A from the total glucose concentration of Sample B.

2.3. Glycogen detection in uterine cells and tissues

Uterine samples were fixed in 10% neutral buffered formalin, dehydrated and mounted in paraffin. Three independent cross sections (4 μ m thick) from each uterus were stained with Period-Acid-Shiff (PAS) reagent to detect glycogen deposits, and counter-stained with hematoxylin. Duplicate sections serving as negative controls were pretreated with diastase (Sigma Chemical Co.) to digest glycogen to glucose, prior to PAS staining. Digital images were subsequently captured at 25 and 400 $\times$, and analyzed in triplicate for each animal using ImageJ software (1.43h, Wayne Rasband WS, US National Institute of Health, NIH, Bethesda, MD, <http://rsb.info.nih.gov/ij/>). To provide detailed illustrations of positive PAS staining in various uterine cell types as well as endometrial gland lumens, a third set of images were captured at 1000 $\times$, under oil immersion.

Total endometrial and myometrial areas were measured by delineating each area at 25 $\times$ with an Intuos electronic pen tablet (Wacom Corp., Vancouver, WA, USA) and expressing the area as total pixel count using the ImageJ autothreshold plugin. Detection of glycogen deposits, based on positive PAS staining (magenta color), was accomplished with the ImageJ colour threshold plugin (ver 1.11). The color intensity standard for glycogen deposits was established using PAS-stained rat liver sections. An average of 10 rat liver color intensity measurements for positive PAS staining were obtained and the mean value of these measurements was used as the standard color threshold against which all uterine sections were analyzed. All PAS staining intensity values after diastase treatment were subtracted from those without diastase, when calculating glycogen content.

The total glycogen content of the endometrium and myometrium were determined for complete uterine cross sections at 25 $\times$, using ImageJ and expressed as total PAS positive pixel counts. To determine glycogen content of glandular and luminal epithelia, required viewing images at 400 $\times$. Because of steroid-induced enlargement of the uterus, the number of glands that could be detected at 400 $\times$, varied from as few as six per section in estradiol-treated mink to as many as 20/section in control mink. We therefore normalized glycogen content values for the glandular and luminal epithelia by dividing the total number of positive PAS pixel counts for glycogen by the total pixel count for the area sampled and expressed the data as percent glycogen content (Table 4). Data are also presented as relative fold-increase compared to controls.

2.4. Total RNA Isolation

Total RNA was isolated from 25 to 50 mg uterine tissue from each mink using the QIAGEN RNeasy® Fibrous Tissue Mini Kit (74704, QIAGEN, Valencia, CA, USA). 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 ratios of 1.9 or greater were used for qPCR analysis.

2.5. Primer design for quantitative polymerase chain reaction (qPCR)

Since nucleotide sequences for mink target genes are unknown, we compared each target gene sequence in the rat with those of other species using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI; NIH <http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome>). Regions of the rat genome for glycogen synthase (Gys), glycogen phosphorylase (Pyg) and glycogen synthase kinase-3B (Gsk3B) that were 95–100% homologous for many species (i.e., mouse, dog, and human), were used to design primers for qPCR (Table 1), which were purchased from Integrated DNA Technologies (Coralville, IA, USA).

2.6. Production of first strand transcripts

Conversion of unstable RNA to stable cDNA transcripts (first strand transcripts) was achieved using a reverse transcriptase generated from the Moloney Murine Leukemia Virus (Promega, Madison, WI, USA), and random hexamer primers, according to the manufacturer's instructions.

2.7. Quantitative PCR (qPCR)

Reactions were carried out in triplicate using Fast Start SYBR Green Master Mix (04-673-514-001; Roche Applied Science, Indianapolis, IN, USA), containing forward and reverse primers at 4 μ M each + cDNA template at 100 to 200 ng. Each sample was subjected to 40 alternating cycles of a three-segment amplification program: 1) 15 s denaturation at 95 ° C; 2) annealing for 1 min at 55 ° C (Gsk3B and B-Actin) or 60 ° C (Gys and Pyg); and 3) elongation at 72 ° C for 1 min. The PCR products (amplicons) were detected in real time, by measuring SYBR-green fluorescence during the annealing stage, with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Efficiencies of amplicon doubling during each PCR cycle were: B-Actin: 100%, Gsk3B: 82%, Gys: 99% and Pyg: 83%. Negative controls consisted of samples with no template and amplification was never above background, indicating a lack of non-specific amplification. Primer specificity was validated by melt-curve analysis for each amplicon, which yielded a single melting temperature for each gene product (Table 1).

Data were analyzed using the Relative Standard Curve Method for Quantification of gene products (Applied Biosystems Chemistry Guide for Real Time PCR, NO. 4348358). Standard curves specific for each amplicon, were generated from three pooled control mink uteri. The cDNA from these uteri was diluted (1:10; 1:100; 1:1000; and 1:10,000), or undiluted and used to construct a standard curve representing the natural log cDNA ng/mL versus Cycle Threshold (Ct) values for each gene product ($r^2 = 0.98$ to 0.99). The slopes for these relationships were: B-Actin, -3.32; Gsk3B, -4.65; Gys, -3.37 and Pyg, -4.5. The amount of each target gene amplification product (amplicon) was expressed in ng/mL and normalized by dividing by the corresponding amount of B-Actin gene product. Data were averaged by treatment group, and expressed in terms of a relative fold-difference compared to controls.

2.8. Western blot analysis (WBA)

A sample from each uterus (50 mg) was homogenized in RadioImmuno Precipitation Assay Buffer (Pierce Biotechnology, Rockford IL, USA). Protein concentration was determined using BioRad protein assay kits (500–0006; BioRad, Hercules, CA, USA). Approximately 36 μ g of protein from each sample was separated into proteins of varying molecular weights by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane using an Xcell II blot module (Invitrogen, Carlsbad, CA, USA), and blocked for 2 h in a 5% milk buffer containing Tween 40 (AC33414-2500; Fisher Scientific) to reduce non-specific binding.

Membranes were incubated with primary antibodies specific for Gys3B (9315; Cell Signaling Technology, Danvers, MA, USA), phospho-Gys3B (p-Gsk3B, 9336S) or B-Actin (4967) at 1:1000 for 24 h at 4 ° C. Blots were subsequently incubated with secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase; 7074; Cell Signaling Technology) at 1:2000 for 2 h at 4 ° C. Blots were visualized by chemiluminescence using a luminol peroxidase kit (PI-34083, Pierce Chemical Company) and captured on photographic film (34092, Fischer Scientific). The relative amount of protein within the blots was quantified by densitometry using Un-Scan IT software (Silk Scientific Inc., Orem UT, USA), and expressed as the number of pixels per unit area, with all samples assayed in duplicate.

2.9. Statistical analysis

Only uteri from mink that survived to the completion of the experiment (Control = 6; E2 = 5, 4-OHE2 = 6 and 2-OHE2 = 4) were analyzed. Lack of tissue for some groups resulted in reduced sample sizes for WBA (4-OHE2 = 3; Control = 4). Comparisons between treatments and controls were made using one-way ANOVA, followed by Bonferroni's post test (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant at $P \leq 0.05$. Statistical differences between the endometrium and myometrium, and glandular and luminal epithelia for individual treatments were determined using unpaired Student's *t*-test ($P \leq 0.05$).

3. Results

Uterine weights of ovariectomized mink were increased approximately 4-fold by estradiol and 3-fold by 4-OHE2 and 2-OHE2 ($P \leq 0.05$) compared to controls, with no difference in uterine weights between 2-OHE2 and 4-OHE2 treated mink (Table 2). Endometrial and myometrial areas were increased 3- and 4-fold respectively, above controls by all three steroids (Table 2, Fig. 1; $P \leq 0.05$). The myometrial area was significantly greater than the endometrial area in control and all steroid-treated animals.

Glycogen concentrations in whole uterine homogenates were increased approximately 10-fold above controls by all three steroids (Table 3, $P \leq 0.05$). Total endometrial glycogen content, as determined with ImageJ, was increased 20-fold by 2-OHE2, 47-fold by 4-OHE2 and 62-fold by estradiol (Fig. 1, $P \leq 0.05$). Total myometrial glycogen content was increased 80-fold by estradiol, 90-fold by 4-OHE2, and 107-fold by 2-OHE2 ($P \leq 0.05$). The total glycogen content did not differ between the endometrium and myometrium in control mink, whereas all three steroids increased total glycogen content of the myometrium compared to the endometrium ($P \leq 0.05$). When normalized for differences in size (area) the percent glycogen content of the endometrium was greater than the myometrium in estradiol-treated mink (5.13 ± 0.35 vs 3.45 ± 0.27 ; $P \leq 0.05$), but not in controls (0.24 ± 0.026 vs 0.16 ± 0.022). The percent glycogen content was greater in the myometrium than endometrium in response to 2-OHE2 (4.83 ± 0.43 vs 1.88 ± 0.50 ; $P \leq 0.05$) and there was no difference in response to 4-OHE2 (4.36 ± 0.44 vs 3.94 ± 0.31).

The percent glycogen content of the glandular epithelia was increased 11-fold by 2-OHE2, 37-fold by 4-OHE2 and 47-fold by estradiol ($P \leq 0.05$) above controls (Fig. 1, Table 4). Percent glycogen content of the luminal epithelia was increased 3-fold by 2-OHE2, 13-fold by 4-OHE2 and 14-fold by estradiol ($P \leq 0.05$) above controls. The percent glycogen content did not differ between the glandular and luminal epithelia of control mink. Treatment with estradiol or 4-OHE2 resulted in a larger percent glycogen content in the glandular than luminal epithelia ($P \leq 0.05$).

Expression of Gsk3B mRNA by the mink uterus was reduced approximately 25% by estradiol and 2-OHE2 and 50% by 4-OHE2 ($P \leq 0.05$; Fig. 2). At the protein level, WBA revealed no difference in the amount of Gsk3B (active) in response to any treatment, nor between hormone treatments and controls (Fig. 3). Expression of p-Gsk3B (inactive) protein was barely detectable in ovariectomized untreated mink. Exogenous 2-OHE2 and estradiol increased production of p-Gsk3B protein 30 and 45-fold ($P \leq 0.05$), respectively, while 4-OHE2 increased production 5-fold, above controls ($P \leq 0.05$). Expression of Gys mRNA by the mink uterus was increased by both CE's ($P \leq 0.05$), but was unaffected by estradiol (Fig. 2). Uterine Pyg mRNA expression was reduced by all three steroids ($P \leq 0.05$).

4. Discussion

Growth of the mink uterus was increased approximately 4-fold by estradiol and 3-fold by 4-OHE2 and 2-OHE2 (Table 2, Fig.1). The myometrium was enlarged 4-fold and endometrium 3-fold by all three steroids. These responses may reflect the large dose of hormones utilized, but could be species specific. In the immature rat, estradiol and 4-OHE2 at 10 ng/d, produced equivalent uterine weight gains, whereas 1000 to 10,000 ng/d of 2-OHE2 were required to produce comparable weights [31]. Treatment of CD-1 mice with these steroids at 2 μ g/d during the first 5 d of life resulted in uterine weight gains of 133% (estradiol), 213% (4-OHE2) and 107% (2-OHE2) [32].

Whole uterine glycogen concentrations, as well as total endometrial and myometrial glycogen content, were significantly increased by estradiol and both catecholestrogens (Table 3, Fig. 1). Although total glycogen reserves were greater in myometrium than endometrium, this appeared to be due in part, to the larger size of the myometrium (Table 2, Fig.1). The percent glycogen content of the glandular epithelia exceeded that of the luminal epithelia, in response to estradiol and 4-OHE2 ($P \leq 0.05$), but not 2-OHE2 (Table 4, Fig. 1). These findings agreed with those reported for other species, showing that exogenous estradiol stimulates uterine glycogen synthesis [18–21], and that 4-OHE2 normally has greater uterotrophic potency than 2-OHE2 (22–24). Large glycogen deposits within the glandular epithelia has been observed by others [5–7], and strengthens the hypothesis that glycogen is an important product of endometrial glands.

Expression of the Gsk3B gene by the mink uterus was reduced by all three steroids (Fig. 2), which could have contributed to increased glycogen synthesis. And yet, our WBA revealed no difference in Gsk3B (active) protein expression between hormone treatments and controls (Fig. 3). It is not known if Gsk3B expression differs between mink uterine endometrium and myometrium, or between glandular and luminal epithelia. If such differences exist, they could have been masked by our analyses of whole-organ homogenates. In support of this hypothesis, Gunin et al., [33–35] demonstrated Gsk3B protein immunostaining in mouse uterine luminal and glandular epithelia, but not stroma. Furthermore, treatment with estradiol resulted in a greater level of total Gsk3B immunostaining than in controls. Salameh et al., [36] showed that the highest human uterine Gsk3B levels occurred in the endometrium, especially the glandular epithelia. We concluded that, although estradiol and both catecholestrogens reduced Gsk3B gene expression, they had no effect on Gsk3B protein formation.

Uterine expression of p-Gsk3B in control mink was barely detectable (Fig. 3). Exogenous 2-OHE2 and estradiol increased production of the protein 30 and 45-fold respectively, whereas 4-OHE2 increased production only 5-fold above controls. It was surprising that 2-OHE2, considered to be less estrogenic than 4-OHE2, had a greater effect on Gsk3B phosphorylation; this could represent a specific role for 2-OHE2 in mink uterine glycogen metabolism. Low p-Gsk3B protein expression by the uterus of control mink agreed with the

findings of Chen et al. [16], who reported that ovariectomized CD-1 mice failed to produce p-Gsk3B, whereas treatment with estradiol increased production of the protein within 2 h. Wang et al. [37], using the ovariectomized cyclin D1 null mouse, could only detect p-Gsk3B at levels slightly above background in the uterus, whereas treatment with estradiol, produced a very strong signal for the protein within 5 h. Sanz et al. [38] administered estradiol to ovariectomized rats, which resulted in a sustained phosphorylation of Gsk3B in the prefrontal cortex. Salameh et al. [36] showed that although Gsk3B mRNA and Gsk3B protein expression did not change throughout the human menstrual cycle, expression of p-Gsk3B increased more than 5-fold during the secretory phase, peaking after ovulation on d 17, just prior to the expected time of implantation. We concluded that increased glycogen accumulation in the mink uterus was due in part, to the phosphorylation and inactivation Gsk3B, in response to estradiol and catecholestrogens, especially 2-OHE2. Expression of Gys mRNA by the mink uterus was increased by 2-OHE2 and 4-OHE2 ($P \leq 0.05$) but interestingly, was unaffected by estradiol (Fig. 2). To the best of our knowledge, all previous investigations on the effects of estrogens on uterine Gys have focused on enzyme activity and not gene expression [39–41]. We concluded that estradiol stimulated uterine glycogen synthesis by enhancing Gys activity, whereas Gys gene expression was increased by catecholestrogens. Thus, the concomitant actions of estradiol and catecholestrogens on the uterus may be requisite for optimal glycogen synthesis in the mink.

Mink uterine Pyg mRNA expression was reduced by estradiol, 2-OHE2, and 4-OHE2 (Fig. 2; $P \leq 0.05$). We are unaware of any published data on uterine Pyg gene expression, whereas there are numerous reports on Pyg activities [40,41]. Nevertheless, our findings agreed with those for the human, where uterine Pyg activity was lowest during the proliferative phase when estrogen concentrations were high [40,41]. In ovariectomized rats, a single sc injection of estradiol reduced uterine Pyg activity at 12 h, which gradually increased from 48 to 96 h [39]. It is likely that reduced Pyg mRNA expression in the mink uterus resulted in decreased p-Pyg protein expression, contributing to greater glycogen reserves. However, because commercial antibodies were unavailable for WBA of Pyg and p-Pyg, we were unable to make this determination. We suspected that some conversion of exogenous estradiol to catecholestrogens would have occurred in the mink uterus. However, if this happened, the amounts were either very small and/or were catabolized at a rate that prevented any observable effects. Regardless, we have demonstrated expression of the CYP1B1 gene, that codes for the enzyme that produces 4-OHE2 [22,32], by the mink uterus and shown that exogenous estradiol doubles expression of the gene (unpublished data; [42]). These findings support those of Paria et al. [24] who showed that treatment of ovariectomized mice with estradiol stimulated uterine 2, 4 hydroxylase activity. Moreover, 4-hydroxylase activity increased during the afternoon of Day 4, just prior to implantation, then declined on Day 5. They proposed that the increase in 4 hydroxylase activity might be due to the increase in preimplantation ovarian estrogen secretion that takes place around noon on Day 4. Similarly in the mink, we envisioned that catecholestrogen production by the uterus may increase during proestrus, mating and implantation when circulating estradiol concentrations were elevated [43–45].

It is unresolved whether uterine glycogen reserves are utilized only after glycogen is catabolized to glucose, or if in addition, embryos take up glycogen from the uterine histotroph by endocytosis. Preimplantation embryos of many species are unable to metabolize glucose until the early blastocyst stage, co-incident with differentiation of the epithelial trophectoderm [46–48]. At that time, embryos switch from oxidizing pyruvate and lactate to glucose metabolism, which appeared to be essential for further development. During blastocyst activation and implantation, uterine histotroph production increased and the trophectoderm took up macromolecules, apparently including glycogen [6,49–51]. We occasionally observed positive PAS staining within the lumens of the mink endometrial

glands (Fig. 1); we inferred that glycogen was being secreted into the uterine lumen. The trophoblast of carnivores becomes extremely phagocytic as implantation progresses and the trophoctoderm is capable of engulfing particles as large as whole erythrocytes [52]. It should not be surprising that embryos may derive glucose by direct uptake of the monosaccharide as well as through phagocytosis of glycogen from the uterine histotroph. In summary, we concluded that glycogen, synthesized in response to estradiol, was an important nutrient produced by the uterine endometrial glands of mink. Since 4-OHE2 and 2-OHE2 but not estradiol increased *Gys* gene expression, we inferred that the effects of estradiol on uterine glycogen synthesis were mediated in part through catecholestrogens. Although circulating estradiol concentrations in mink declined during estrus and pregnancy, their concentrations were still much higher than during the anestrous. As a consequence, we propose that estradiol and catecholestrogens will continue to promote uterine glycogen synthesis during these intervals. In that regard, such a mechanism could teleologically serve to divert circulating glucose to the uterus for local storage and utilization during embryo development, implantation, and perhaps pregnancy.

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Fig. 1.

Uterine cross-sectional images of mink treated with estradiol, 4- hydroxycatecholestradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2) or as controls. All sections (4 μm thick) were stained with PAS and counterstained with hematoxylin. Images were captured at 25× (left column), 400× (middle column) and 1000× (right column). Small arrows identify areas of positive PAS staining in uterine cells. Large arrows identify positive PAS staining within the glandular lumen. M = myometrium, E = endometrium, GE = glandular epithelium, LE = luminal epithelium.

Fig. 2.

Relative gene expression levels for glycogen synthase kinase-3B, glycogen synthase and glycogen phosphorylase (mean $\pm$ SEM) by the uteri of ovariectomized mink treated with estradiol (N=5), 4-hydroxycatecholestradiol (4-OHE2; N=6), 2- hydroxycatecholestradiol (2-OHE2; N=4) or as controls (N=6).

^{a,b}Groups without a common letter differ (P<0.05).

Fig. 3.

Western blot analyses (WBA) for glycogen synthase kinase-3B (Gsk3B) and p-Gsk3B (Gsk3B) proteins (mean $\pm$ SEM) expressed by the uteri of ovariectomized mink treated with estradiol (N=5), 4-hydroxycatecholestradiol (4-OHE2; N=3), 2- hydroxycatecholestradiol (2-OHE2; N=4) or as controls (N=4).

^{a-c}Within a protein, groups without a common letter differ (P<0.05).

Table 1

Primers used for qPCR of mink uterine gene transcripts and amplicon characteristics.

Genes	Forward & reverse primers (5' to 3')	Amplicon (bp)	Melt temperature (°C)	Accession No.*
Gsk3B	CTTGCGGGAGAACTAATGCTG CCAATGACTTTAGTGTCTGTGTAAGTCTG	295	83.3 ± 0.03	NM_032080
Pyg	GTCAGAACAGATCTC CAC TGCTGG GTCTTTGAAGAGGTCTGGCTGATTGG	296	81.5 ± 0.09	NM_012638.1
Gys	AGTCCTCAGAGCAGC GATGTGG GTACCATCACAGTACGGTGACACATG	327	77.4 ± 0.23	NM_001109615.1
B-Actin	GATGACC CAGATCATGTTCGAG CCATCTCCTGCTCGAAGTCC	329	86.0 ± 0.03	NM_031144

Abbreviations: glycogen synthase (*Gys*), glycogen phosphorylase (*Pyg*), glycogen synthase kinase-3B (*Gsk3B*), beta actin (*B-Actin*).

* <http://www.ncbi.nlm.nih.gov>

Table 2

Mean ($\pm$ SEM) uterine weights and endometrial and myometrial areas of ovariectomized mink treated with estradiol, 4-hydroxycatecholestradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2), or as controls. Relative fold-increase above controls are given in parentheses.

Group	No. mink	Uterine weight (mg)	Endometrial area, (pixels $\times 10^4$)	Myometrial area, (pixels $\times 10^4$)
Control	6	322.23 $\pm$ 42.33 ^a	3.68 $\pm$ 0.34 ^{Aa}	6.41 $\pm$ 0.35 ^{Ba}
Estradiol	5	1352.10 $\pm$ 145.47 ^b (4)	11.07 $\pm$ 0.67 ^{Ab} (3)	24.17 $\pm$ 1.26 ^{Bb} (4)
4-OHE2	6	854.56 $\pm$ 101.51 ^c (3)	9.86 $\pm$ 1.01 ^{Ab} (3)	23.58 $\pm$ 1.66 ^{Bb} (4)
2-OHE2	4	826.75 $\pm$ 107.39 ^c (3)	9.82 $\pm$ 3.05 ^{Ab} (3)	22.83 $\pm$ 1.60 ^{Bb} (4)

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

^{A,B} Within a row, mean endometrial and myometrial values without a common superscript differ ($P \leq 0.05$).

Table 3

Mean $\pm$ SEM glycogen concentrations in whole uteri, and total glycogen content for endometrium and myometrium of ovariectomized mink treated with estradiol, 4-hydroxycatecholestradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2), or as controls. Relative fold-increase above controls are given in parentheses.

Group	No. mink	Glycogen (mg/g dry weight)	Endometrial glycogen (pixels $\times 10^3$)	Myometrial glycogen (pixels $\times 10^3$)
Control	6	4.58 $\pm$ 1.16 ^{aa}	0.09 $\pm$ 0.01 ^{Aa}	0.10 $\pm$ 0.02 ^{Aa}
Estradiol	5	43.79 $\pm$ 5.35 ^b (10)	5.68 $\pm$ 0.44 ^{Ab} (62)	8.33 $\pm$ 0.85 ^{Bb} (80)
4-OHE2	6	48.64 $\pm$ 4.02 ^b (10)	4.30 $\pm$ 0.40 ^{Ab} (47)	9.30 $\pm$ 0.80 ^{Bb} (90)
2-OHE2	4	41.36 $\pm$ 3.23 ^b (10)	1.85 $\pm$ 0.41 ^{Ac} (20)	11.0 $\pm$ 1.18 ^{Bb} (107)

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

^{A,B} Within a row, mean endometrial and myometrial values without a common superscript differ ($P \leq 0.05$).

Table 4

Mean $\pm$ SEM percent glycogen content for glandular and luminal epithelia of ovariectomized mink treated with estradiol, 4-hydroxycatechol estradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2), or as controls. Relative fold-increase above control values are given in parentheses.

Groups	No. mink	Glandular epithelia	Luminal epithelia
Control	6	0.45 $\pm$ 0.15 ^{Aaa}	0.50 $\pm$ 0.14 ^{Aaa}
Estradiol	5	21.11 $\pm$ 4.14 ^{Ab} (47)	7.36 $\pm$ 0.85 ^{Bb} (14)
4-OHE2	6	16.57 $\pm$ 3.67 ^{Ab} (37)	6.67 $\pm$ 0.79 ^{Bb} (13)
2-OHE2	4	5.08 $\pm$ 2.75 ^{Ac} (11)	1.44 $\pm$ 0.28 ^{Ac} (3)

^{a-c} Within a column, means with different superscripts differ ($P \leq 0.05$)

^{A,B} Within a row, means with different superscripts differ ($P \leq 0.05$).