BASIC SCIENCE: GYNECOLOGY

Enhanced estrogen-induced proliferation in obese rat endometrium

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OBJECTIVE: We tested the hypothesis that the proliferative estrogen effect on the endometrium is enhanced in obese vs lean animals.

STUDY DESIGN: Using Zucker fa/fa obese rats and lean control, we examined endometrial cell proliferation and the expression patterns of certain estrogen-regulated proproliferative and antiproliferative genes after short-term treatment with estradiol.

RESULTS: No significant morphologic/histologic difference was seen between the obese rats and the lean rats. Estrogen-induced proproliferative genes cyclin A and c-Myc messenger RNA expression were significantly higher in the endometrium of obese rats compared with those of the lean control. Expression of the antiproliferative gene p27Kip1 was suppressed by estrogen treatment in both obese and lean rats; however, the decrease was more pronounced in obese rats. Estrogen more strongly induced the antiproliferative genes retinaldehyde dehydrogenases 2 and secreted frizzled-related protein 4 in lean rats but had little or no effect in obese rats.

CONCLUSION: Enhancement of estrogen-induced endometrial proproliferative gene expression and suppression of antiproliferative gene expression was seen in the endometrium of obese vs lean animals.

Key words: endometrial, estrogen, obesity, proliferation

Obesity affects more than 25% of adult women in the United States and continues to increase in prevalence. Numerous epidemiologic studies have demonstrated that obesity is a major risk factor for endometrial cancer.[1] Although an average woman has a 3% lifetime risk

of endometrial cancer, obese women have a 9-10% lifetime risk of endometrial cancer.[2] The increased peripheral conversion in adipose tissue of adrenal steroids to estrone and the increased bioavailability of free estrogens because of decreased sex hormone-binding globulin contribute to a "hyperestrogenic state" in obese women, which results in increased endometrial cell proliferation, leading to endometrial hyperplasia and cancer. Clinical studies have shown that patients with endometrial cancer exhibit higher plasma levels of estrogens vs controls.[3] However, in a large study by Potischman et al,[4] the authors found that obesity remained a significant risk factor for the development of endometrial cancer even after controlling for endogenous estrogens. These results, and those of others, suggest that excessive estrogen alone cannot fully explain the association between obesity and endometrial cancer. Insulin resistance, associated with obesity, may enhance the effect of estrogen in the endometrium.[5]

Acting via its receptor, estrogen promotes cell proliferation through regulating the expression of a wide variety of target genes. Studies by our group and others have shown that estrogen induces endometrial proproliferative and antiproliferative gene expression.[6] Among the estrogen-regulated genes, the expression of proliferative gene cyclin A and c-Myc are up-regulated by estrogen in the endometrium,[6,7] and their expression is highly correlated with the entrance of cells into the S-phase,[8,9] and linked to cellular proliferation or tumorigenesis.[10] Expression of p27Kip1, a potent negative regulator of cell cycle and cellular proliferation, is inhibited by estrogen in the endometrial cell.[11] A progressive decrease in p27Kip1 expression from normal, through hyperplastic endometrium, to endometrial carcinoma has been reported.[12] Progesterone receptor (PR), secreted frizzled-related protein 4 (sFRP4), and retinaldehyde dehydrogenases 2 (RALDH2) are estrogen-regulated antiproliferative genes whose expression and activity are up-regulated by estrogen in the endometrum.[13-16]

In this study, we examined the effect of estrogen on endometrial cell proliferation in the Zucker fa/fa rats. The Zucker fa/fa rats exhibit many of the pathophys-

iologic features present in obese humans, including severe obesity, chronic insulin resistance, and hyperinsulinemia.[17-19] We examined the effect of estrogen on the expression of proliferative genes cyclin A and c-Myc, and antiproliferative genes p27Kip1, PR, sFRP4, and RALDH2. In addition, we also compared estrogen-induced activation of Akt and extracellular signal-regulated protein kinase 1/2 (Erk1/2) mitogen-activated protein kinase (MAPK) signaling in obese and lean rats.

MATERIALS AND METHODS
Animals
Mature (5-week-old) female Zucker fa/fa rats and their lean littermates (Harlan Laboratories, Indianapolis, IN) were housed in plastic cages on a 12:12 light/dark cycle with free access to water and food (Nestle-Purina, St Louis, MO). After 1 week of acclimation, animals were ovariectomized, held for 5 days to clear endogenous ovarian hormones, and then injected subcutaneously with either 17β-estradiol (E2, 40 μg/kg) or vehicle (5% ethanol) once daily for 3 consecutive days. Five to 6 animals were used in each group, except as mentioned. The following day, all rats were killed. For RNA analysis, the uterine tissue was scraped and flash frozen in liquid nitrogen and stored at -80°C.

Plasma glucose level and insulin level detection
Three fat and 3 lean rats were used in each group. All rats were fasted overnight and were subjected to an oral glucose tolerance test the next morning. Plasma glucose and insulin levels were measured at 30, 60, and 120 minutes after glucose challenge (2 g glucose/kg bodyweight). Plasma glucose concentration was measured using the Ascensia Coutour Blood Glucose Monitoring System (Bayer, Inc, Mishawaka, IN). Insulin levels were measured using radioimmunoassay by LINCO Diagnostic Services, Inc (St Charles, MO).

Immunohistochemistry
For immunohistochemical detection of BrdU (Bromodeoxyuridine) incorporation, all rats had intraperitoneal injection of BrdU at a dose of 100 mg/kg of bodyweight. Rats were killed 90 minutes after BrdU injection, and fresh uterine tissue was fixed in neutral-buffered 10% formalin and paraffin embedded. Paraffin-embedded sections of rat uteri were cut at 4-μm thickness, deparaffinized with xylene, and rehydrated with graded ethanol. BrdU immunostaining was performed using the BrdU in-situ detection kit (BD Biosciences, San Diego, CA). The slides were counterstained with Mayer's hematoxylin for 1 minute. The total number of BrdU-staining nuclei was counted in 10 randomly selected fields.

For immunohistochemical detection of phosphorylated-Akt (pAkt) and p27, endogenous peroxidase activity was inactivated using 3% hydrogen peroxide. After blocking for 30 minutes in 10% horse serum, the sections were incubated in primary antibody against pAkt Ser[473] or p27 (Cell Signaling, Danvers, MA; 1:200) overnight at 4°C, followed by the incubation with biotinylated antirabbit immunoglobulin G (IgG) and streptavidin-horseradish peroxidase (HRP) (Dako Corporation, St Louis, MO). Diaminobenzidine solution was applied to visualize the complex. The sections were counterstained with Mayer's hematoxylin. To evaluate differential expression levels of pAkt, the following 4-point scale scoring system was used: 0, negative staining; +1, weak staining; +2, moderate staining; +3, strong staining. Slides were read by 2 investigators.

RNA isolation and real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)
Total RNA was extracted from frozen endometrial tissue using Tri-reagent (Molecular Research Center, Inc, Cincinnati, OH), as described previously. [20] Briefly, tissues were homogenized in Tri-reagent (Sigma-Aldrich, St Louis, MO), RNA was precipitated with isopropanol from the aqueous phase, and the resuspended RNA plus EtOH was applied to an RNeasy spin column (Qiagen, Valencia, CA). RNA was eluted, treated with DNase I, and stored at -80°C.

For each transcript, specific polymerase chain reaction (PCR) primer pairs and a dual fluorochrome-labeled hybridization probe (TaqMan probe) were designed using Primer Express (Applied Biosystems, Foster City, CA) or Beacon Designer (Premier Biosoft International, Palo Alto, CA) (Table). Five-nanogram aliquots of total RNA were reverse transcribed in quadruplicate (including an RT [−] control for each sample) with 1X RT buffer, 400 nM reverse primer, 10 mM DTT, 500 μM dNTPs, and Superscript II (Invitrogen, Carlsbad, CA) at 50°C for 30 minutes, followed by 72°C for 5 minutes. The PCR master mix was then added directly to each RT reaction (10 μL) and contained 1X PCR buffer, 400 nM forward and reverse primers, 100 nM fluorogenic probe, 5 mM MgCl$_2$, 150 nM SuperROX (Biosearch Technologies, Novato, CA), and 1.25 U Taq Polymerase (Sigma-Aldrich). Amplification was performed using the ABI Prism 7700 or 7900 (Applied Biosytems) at 95°C for 1 minute, followed by 40 cycles of 95°C for 12 seconds and 60°C for 30 seconds. Data were analyzed by using the sequence detection application software; the quantification of transcripts was determined by interpolating the mean C_t value (PCR cycles to threshold) of an unknown sample on a 5-log standard curve run on each plate using 10-fold decrements of a known amount of a synthetic DNA oligo spanning the PCR amplico. All values were corrected for RNA input by normalization to the level of 18S ribosomal RNA (18SrRNA) and are expressed as the percent of 18SrRNA. All real-time RT-qPCR reactions were set up by using liquid handling robotics.

Immunoblots
Immunoblot analysis was performed as previously described.[20] In brief, frozen samples of uterine horn were homogenized in 200 μL ice-cold lysis buffer consisting of 20 mM Tris PH 8.0, 135 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 1% Triton X-100, 10% glycerol, 1 time complete protease inhibitor cocktail, 10 mM NaF, and 5 mM Na$_3$VO$_4$. The homogenates were incubated on ice for 30 minutes, followed by centrifugation at 14,000g at 4°C for 20 minutes. The su-