Enhanced estrogen-induced proliferation in obese rat endometrium

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Abstract

Objective—We tested the hypothesis that the proliferative estrogen effect on the endometrium is enhanced in obese versus 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 pro-proliferative and anti-proliferative genes after short-term treatment with estradiol.

Results—No significant morphological/histological difference were seen between the obese rats and the lean rats. Estrogen-induced pro-proliferative genes cyclin A and c-Myc mRNA expression were significantly higher in the endometrium of obese rats compared with that of the lean control. Expression of the anti-proliferative 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 anti-proliferative genes RALDH2 and sFRP4 in lean rats, but had little or no effect in obese rats.

Conclusion—Enhancement of estrogen-induced endometrial pro-proliferative gene expression and suppression of anti-proliferative gene expression was seen in the endometrium of obese versus lean animals.

Keywords

Obesity; estrogen; endometrial; proliferation

Obesity affects over 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

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Condensation: As compared to the endometrium of lean rats, obese rat endometrium shows an increase in cell proliferation markers with estrogen treatment.

factor for endometrial cancer.[1] While 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 due to 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 versus controls. [3] However, in a large study by Potischman et al, the authors found that obesity remained a significant risk factor for the development of endometrial cancer even after controlling for endogenous estrogens. [4] These results, and that 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 pro-proliferative and anti-proliferative 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 anti-proliferative genes whose expression and activity are up-regulated by estrogen in the endometrum. [13-16]

In the present 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 pathophysiological 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 anti-proliferative 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) 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 (Purina). After one 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 three consecutive days. Five to 6 animals were used in each group except as mentioned. The following day, all rats were sacrificed. 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 lean rats were used in each group. All rats were fasted overnight, and were subjected to an oral glucose tolerance test the following morning. Plasma glucose and insulin levels were measured at 30, 60, and 120 minutes after glucose challenge (2g glucose/kg body weight). Plasma glucose concentration was measured using the Ascensia Coutour Blood

Glucose Monitoring System (Mishawaka, US). Insulin levels were measured using radioimmunoassay by LINCO Diagnostic Services, Inc.

Immunohistochemistry

For immunohistochemical detection of BrdU (Bromodeoxyuridine) incorporation, all rats had intraperitoneal injection of BrdU at a dose of 100mg/kg of body weight. Rats were sacrificed 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 4μm thickness, deparaffinized with xylene, and rehydrated using graded ethanol. BrdU immunostaining was performed using the BrdU in-Situ detection kit (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, MA, 1:200) overnight at 4°C, followed by the incubation with biotinylated anti rabbit IgG and streptavidin-HRP (Dako Incorporation). 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 four-point scale scoring system was used: 0, negative staining; +1, weak staining; +2, moderate staining; +3, strong staining. Slides were read by two investigators.

RNA isolation and real-time quantitative reverse transcription-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, St Lousis, 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 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 Intl., Palo Alto, CA) (Table 1). Five ng aliquots of total RNA were reverse transcribed in quadruplicate [including a RT (-) control for each sample] with 1× 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 1× 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, St. Louis, MO). Amplification was performed using the ABI Prism 7700 or 7900 at 95°C for 1 minute, followed by 40 cycles of 95°C for 12 seconds and 60°C for 30 seconds. Data was analyzed using the Sequence Detection Application software, the quantification of transcripts were 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 and are expressed as the percent of 18SrRNA. All real-time RT-qPCR reactions were set up 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, $1 \times$ complete protease inhibitor cocktail, 10 mM NaF, and 5 mM Na_3VO_4. The homogenates were incubated on ice for 30 minutes, followed by centrifugation at 14,000 g at 4°C for 20 minutes. The supernatants were collected, and protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were resolved by SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat milk, the membranes were probed with primary antibody p27, pAkt, Akt, Erk, phosphorylated (pErk) (Cell Signaling, MA, 1:1000), or β-actin (Sigma, St Lousis, MO, 1:5000) followed by HRP-conjugated secondary antibody. When necessary, PVDF membranes were stripped at 50°C for 30 minutes in stripping buffer (62.5 mM Tris-HCl, PH 6.8, 2% SDS and 100 mM β-mercaptoethanol) for a second round of immunoblotting.

Statistical Analysis

Statistical calculations were performed using SPSS 12.0 software. Statistical significance between groups was evaluated using one-way analysis of variance. Differences between groups were determined by the Turkey test. Results were presented as mean ± SEM with $p < 0.05$ considered to be statistically significant.

Results

Zucker fa/fa rats replicate the features of high body weight and low insulin sensitivity in obese women

Glucose administration induced a high glucose level in the obese animals at all time points, from 0 to 120 minutes after glucose challenge, and the difference was significant at the 30 minutes and 60 minutes time point ($p<0.05$). The plasma insulin level was also significantly higher in the Zucker fa/fa rats at 30 minute and 60 minute time points, as compared to their lean counterparts ($p<0.05$) (Fig. 1), indicating that the obese rats were displaying evidence of insulin resistance.

Endometrial cell proliferation

Estradiol administration increased the uterine wet weight in both lean and obese rats. At the microscopic level, the lean and obese rat uteri were microscopically identical. Following estrogen treatment, both sets of uteri exhibited similar microscopic changes-the uterine epithelium became taller and more columnar. Although we saw a trend of increased luminal epithelial height in estrogenized obese rats compared with the estrogenized lean rats, the difference was not statistically significant (data not shown).

To measure the effect of estradiol administration on endometrial cell proliferation, BrdU was injected intraperitoneally 90 minutes prior to sacrifice. BrdU-labeled cells were absent or minimally detected in epithelial cells of the uterus in both the lean and obese controls, while the BrdU-labeled epithelial cell number was significantly increased in both the lean and obese rats after estradiol treatment (Fig. 2, $p<0.05$). The number of BrdU-labeled cells was higher in the estrogenized obese rat endometrium as compared to their lean counterparts (38.7±20.9 vs. 22±12), but the difference was not statistically significant.

Expression of cell proliferative genes

The basal levels of cyclin A transcripts in the endometrium of lean and obese animals were below the detection of quantitative RT-PCR assay. In the lean rats, estrogen treatment resulted

in a dramatic induction of cyclin A expression in the endometrium. The response to estrogen was even greater in the Zucker fa/fa rats. When the absolute abundance of cyclin A transcripts was compared, the level was approximately 3-fold higher in the estrogen-treated obese rats than in their estrogen-treated lean littermates ($p < 0.05$).

The basal levels of c-Myc transcripts were similar in lean and obese rats. Estrogen treatment had little effect on the expression of c-Myc in lean animals, but significantly increased the expression of c-Myc in obese rats ($p < 0.05$, Fig. 3). In addition, the absolute level of c-Myc transcripts in the endometrium of estrogen-treated obese rat was substantially higher than in the estrogen-treated lean rat (88% increase, $p < 0.05$).

Cell cycle inhibitor p27Kip1 expression

At baseline, without estrogen treatment, there was no difference between the lean and obese rats in the level of p27Kip1 transcripts in the endometrium (Fig. 4). Estrogen treatment in lean rats resulted in a significant decrease in the level of p27Kip1 transcripts (47% of vehicle control, $p < 0.05$, Fig. 4). The effect of estrogen on p27Kip1 expression was even more pronounced in obese rats. The level of p27Kip1 mRNA was decreased to 23% of lean vehicle control ($p < 0.05$) in the obese animals following 3 days of estrogen treatment. When the absolute abundance of p27Kip1 transcripts in the endometrium of estrogen-treated lean and obese rats was compared, p27Kip1 mRNA level in estrogen-treated Zucker fa/fa rats was significantly lower ($p = 0.007$) than that in their estrogen-treated lean littermates.

To further quantify the alterations in p27Kip1 expression, protein lysates from the uterine tissues were immunoblotted with an antibody against the p27Kip1 protein. As shown in Fig 4, estradiol treatment significantly decreased the level of p27Kip1 protein in the uteri from both Zucker obese and lean rats, but the decrease in the obese animals was more pronounced. Immunohistochemical staining with antibody against p27Kip1 on uterine tissue sections demonstrated that p27Kip1 was expressed in the nuclei of endometrial glandular cells, and its expression was significantly suppressed by estradiol treatment in obese and lean animals (data not shown).

Estrogen regulated anti-proliferative gene expression

Progesterone receptor (PR) transcript levels were increased significantly in the endometrium of obese rats after estrogen treatment, but no such estrogen effect was observed in the lean rats (Fig. 5).

Estradiol treatment significantly increased sFRP4 transcript level in the endometrium of lean rats ($p<0.05$), while in the obese rats, the sFRP4 level remained unchanged (Fig. 5).

RALDH2 mRNA expression in the lean rat endometrium was significantly increased compared with the lean vehicle-treated control group ($p<0.05$). In the obese rats, RALDH2 mRNA level in the estradiol-treated group remained unchanged compared to vehicle treated group (Fig. 5).

Estrogen stimulated Akt and MAPK signaling

Compared with the vehicle control groups, estradiol treatment increased the Akt phosphorylation in both lean and obese rats as evidenced by Western blot analysis. A stronger Akt phosphorylation signal was seen in both estrogenized lean and obese animals compared with the vehicle-treated control groups. There was not a significant difference in the Akt phosphorylation between the estrogenized lean and obese rats (Fig. 6). These findings were confirmed by immunohistochemical staining. While stronger pAkt staining was seen in the estrogenized lean and obese animals compared with the vehicle control groups, there was no significant difference between the estrogenized obese and lean animals (data not shown).

Western blot analysis using pErk1/2 antibody demonstrated that estrogen stimulation increased Erk1/2 phosphorylation in both lean and obese animals. Furthermore, a significant increase in estrogen effect on Erk1/2 was seen in the obese endometrium compared to the lean endometrium (Fig. 6).

Comment

Obese women are clearly at an increased risk for developing endometrial cancer. [1, 2] We hypothesized that in addition to hyperestrogenism, obesity-related factors including insulin resistance may potentiate the activity of estrogen. In this study, using genetically obese Zucker fa/fa rats as an experimental model, we evaluated the effects of estrogen on endometrial cell proliferation. In addition, we examined estrogen-induced pro-proliferative and anti-proliferative biomarkers in the endometrium.

Estrogens have been shown to stimulate endometrial cell proliferation in mouse endometrium. [21] Our results confirm this observation in the rat endometrium. In our study, BrdU-labeled cells were dramatically increased in the uteri of both lean and obese rats in the estradiol-treated animals compared with their control groups. When the estrogen-treated obese animals were compared to the estrogen-treated lean animals, there was a trend of increased endometrial proliferation, but it was not statistically significant. A possible explanation was that the difference between the estrogenized lean and obese rat endometrium was too weak to be visualized at the histological level.

Therefore, we examined markers of estrogen-induced pro-proliferation and anti-proliferation at the molecular level. We chose to study the expression of cyclin A and c-Myc based on the following three criteria: (1) the expression of these genes are highly correlated with the entrance of cells into the S-phase, [8, 9] (2) their expression has been linked to cellular proliferation or tumorigenesis, [10] (3) their expression is regulated by estrogen. [6, 7] In line with previous studies, we found that estrogen treatment provoked a dramatic increase in cyclin A transcripts in the endometrium. In addition, we found that the effect was much more pronounced in the Zucker fa/fa rats compared with lean controls. Interestingly, estrogen also induced significant increase in the expression of c-Myc gene in the endometrium of obese versus lean animals. Collectively, these results add further support to our hypothesis that estrogen induces an abnormal ratio of pro-proliferative and anti-proliferative signals in obese rat endometrium, leading to the increased estrogen-induced proliferation.

Disruption of the balance between estrogen-induced proliferative genes and anti-proliferative genes provides another avenue for deregulated cell proliferation. p27Kip1 is a potent negative regulator of cell cycle and cellular proliferation, and is negatively regulated by estrogen in the endometrium. [11] Our results demonstrate that compared to lean animals treated with estrogen, obese rat endometrium treated with estrogen has a significant lower level of p27Kip1, thus predisposing the obese rat to uncontrolled estrogen-driven endometrial proliferation. In the endometrium, a progressive decrease in p27Kip1 expression from normal through hyperplastic endometrium to endometrial carcinoma has been reported. [12] While the increase in estrogen-induced PR was not different between obese and lean animals, other estrogen-induced anti-proliferative markers were differently induced.

sFRP4 modulates Wnt signaling through direct interaction with the Wnt gene. [13] It has shown that sFRP4 mRNA levels were decreased in endometrial tumors versus normal tissue, [22] and sFRP4 has functions of a tumor suppressor. [23] Similar to our results in lean animals, the induction of sFRP4 gene expression by estrogen treatment has been reported in Fujita's study. [13] However, in obese rat endometrium, we saw a decrease in sFRP4 expression. The difference in estrogen stimulation of endometrial sFRP4 expression between the lean and obese animals suggests that estrogen-induced sFRP4 expression was disrupted in obese rats.

RALDH2 expression is regulated by estrogen. Ruhl showed that RALDH2 mRNA expression was rapidly induced by estradiol treatment in the uterus of ovariectomized mice. [24] Deng et al extended this result to human tissue, showing an estrogen-induced RALDH2 mRNA expression in human endometrium.[16] In our study, we also observed an increase in estrogen induced RALDH2 mRNA expression in the lean rat endometrium. However, in the obese rat endometrium, we found the estrogen effect was inhibited, with RALDH2 mRNA expression remaining unchanged after estrogen treatment.

A possible explanation of the increase in endometrial proliferation in obese versus lean animals is that the balance between estrogen-induced pro-proliferative and anti-proliferative endometrial gene expression is disrupted in obese rats. In lean animals, estrogen-induced anti-proliferative genes, such as sFRP4 and RALDH2, counterbalance the estrogen-induced proliferative effect. However, in obese animals, estrogen-induced anti-proliferative gene expression is inhibited, and the inhibitory effect of estrogen on anti-proliferation gene expression is increased, as shown in the expression of p27Kip1. Furthermore, estrogen-induced expression of the proliferative genes is enhanced in the obese animal, as shown by our data of cyclin A and c-Myc. Thus the overall effect is the disruption of the balance between the expression of proliferative genes and anti-proliferative genes in obese rat endometrium which results in increased proliferation.

We demonstrated that estradiol treatment activates phosphatidylinositol 3 kinase (PI3K)/Akt signaling, but its action is not enhanced in the obese animals. However, we did find a difference in Erk MAPK signaling. ERK signaling is synonymous with cell proliferation, although it is now clear that this pathway is also linked to cell differentiation, survival, migration, angiogenesis, and chromatin remodeling [25-27]. Activation of the ERK MAPK signaling is one of the early events of the endometrial cells in response to estrogen stimulation, which further activates the expression of downstream genes, such as c-Myc[28-29]. We provide evidence that estrogen induces Erk1/2 activation to a greater degree in obese rats compared with that in lean animals, suggesting that rather than activation of the Akt pathway, activation of Ras/Raf/MAPK signaling might be one of the underlying pathways leading to the pronounced proliferative effect of estrogen in endometrium of obese animals.

In summary, we demonstrated that estrogen-induced expression of pro-proliferative signals cyclin A and c-Myc are increased in obese rat endometrium compared with lean rat endometrium. In addition, we demonstrated suppression of the anti-proliferation gene p27Kip1, and an inhibition of estrogen-induced anti-proliferation genes sFRP4 and RALDH2. Erk1/2 MAPK phosphorylation is significantly increased in the estrogen-treated endometrium of obese as compared to lean animals. These results suggest that the proliferative effect of estrogen is enhanced, so that the balance of pro-proliferative and anti-proliferative is disrupted in the obese state.

Acknowledgments

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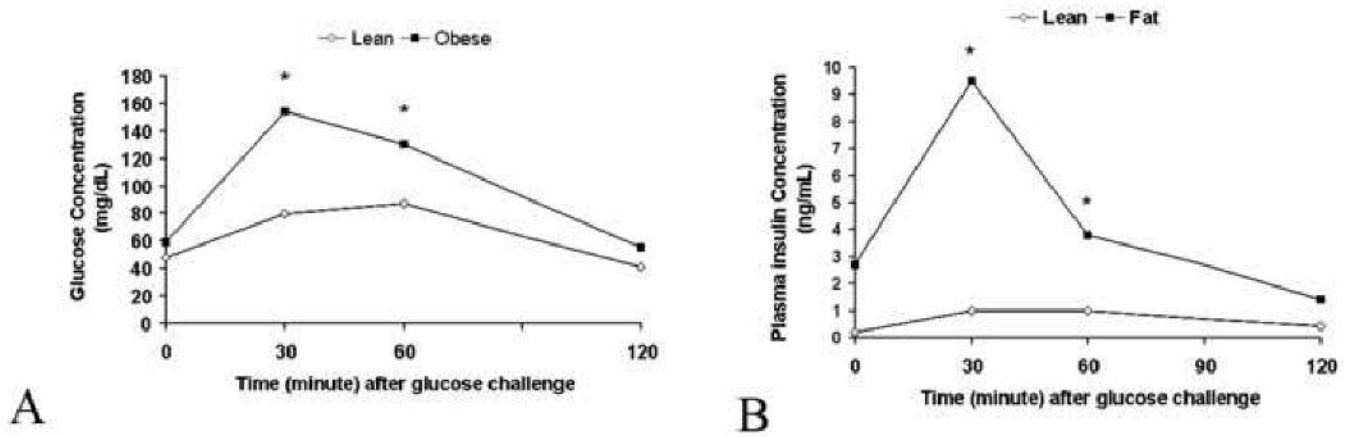
Figure 1. Oral glucose tolerance test and plasma glucose and insulin levels in zucker obese and lean rats

Animals were fasted overnight before glucose challenge (2g/kg body weight). Plasma glucose (A) and insulin (B) level were determined before and up to 120min after glucose challenging. * p<0.05, n=3
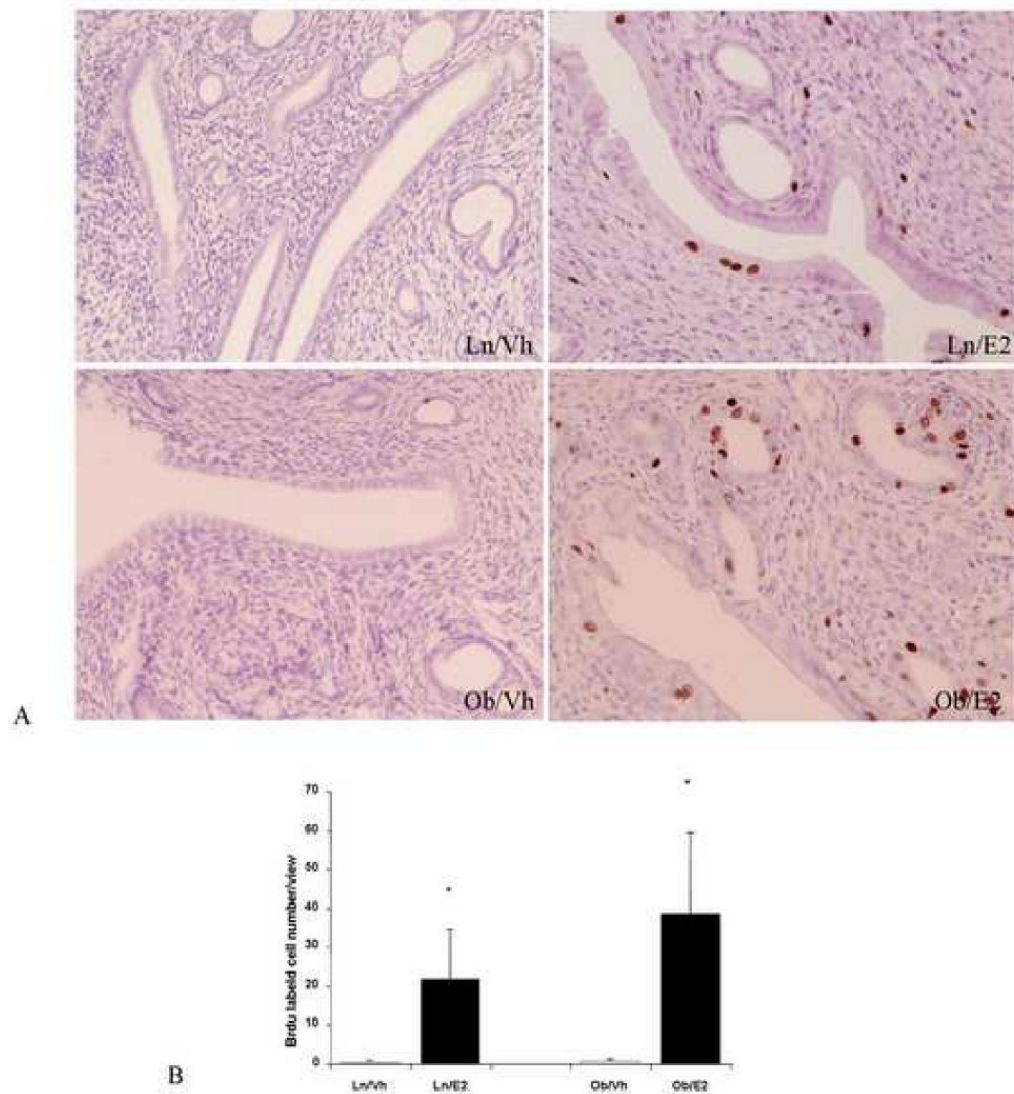
Figure 2. Comparison of endometrial cell proliferation in zucker obese and lean rats
Immunohistochemical staining of BrdU labeled endometrial cell. BrdU (100mg/kg) were IP injected to rats 90 minutes before they were sacrificed. Uteri tissue sections were stained with anti-BrdU antibody. 10 views were counted for BrdU labeled nuclei in each slide. * $p < 0.05$, n=6
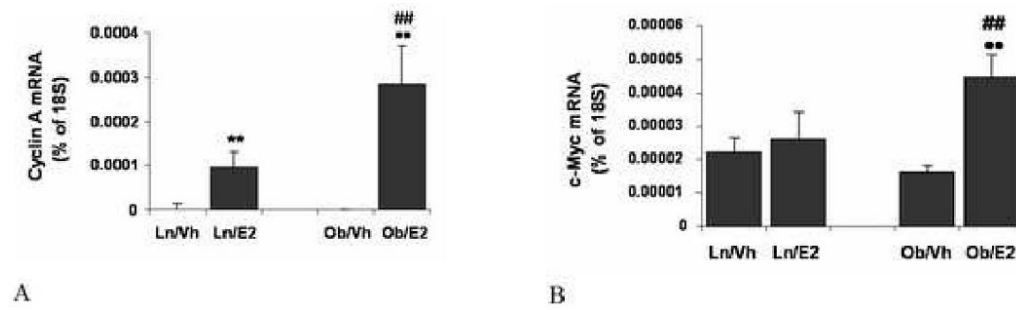
Figure 3. Expression of pro-proliferative genes in the Rat Endometrium
The transcript levels of Cyclin A (A), PCNA (B), and c-Myc (C) in the endometrium from ovariectomized zucker fa/fa rats (Ob) and their lean littermates (Ln) receiving vehicle (Vh) or 17β-estradiol (E2) for 3 days were measured by real-time quantitative RT-PCR. Each sample was assayed in triplicate. The numbers of molecules of cylin A, PCNA and c-Myc in 20 ng total RNA were normalized to and expressed as the percentage of 18S. n = 5~6. **, $p < 0.05$ compared with Ln/Vh; ••, $p < 0.05$ compared with Ob/Vh; ##, $p < 0.05$ compared with Ln/E2.
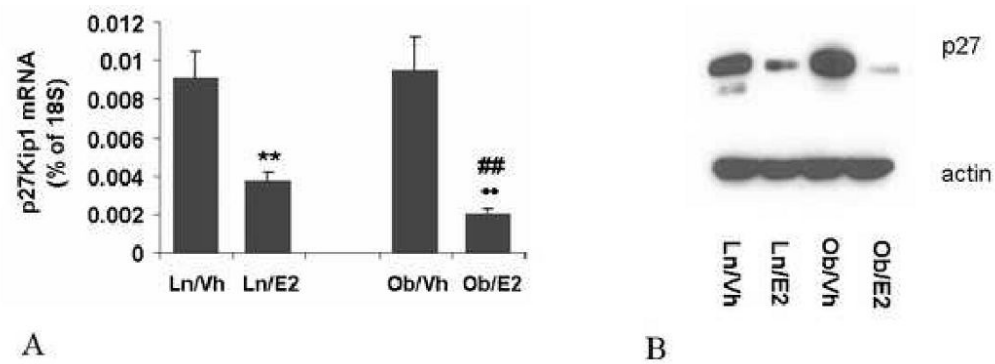
Figure 4. Expression of p27Kip1 in the rat endometrium
(A) Immunoblots of rat endometrium lysates against p27Kip1 antibody. 25 ug of protein from the uteri was subjected to immunoblotting with anti- p27Kip1 antibodies. The blots were then stripped and reprobed with anti- actin antibodies. 1. Lean control; 2. Lean E2; 3. Obese control; 4. Obese E2 (B) real-time quantitative RT-PCR analysis of p27Kip1 mRNA level in rat endometrium. 20 ng of endometrial RNA was assayed in triplicate for p27Kip1 transcripts. Absolute values were normalized to and expressed as a percentage of 18S. n = 5~6. **, $p < 0.05$ compared with Ln/Vh; ••, $p < 0.05$ compared with Ob/Vh; ##, $p < 0.05$ compared with Ln/E2.
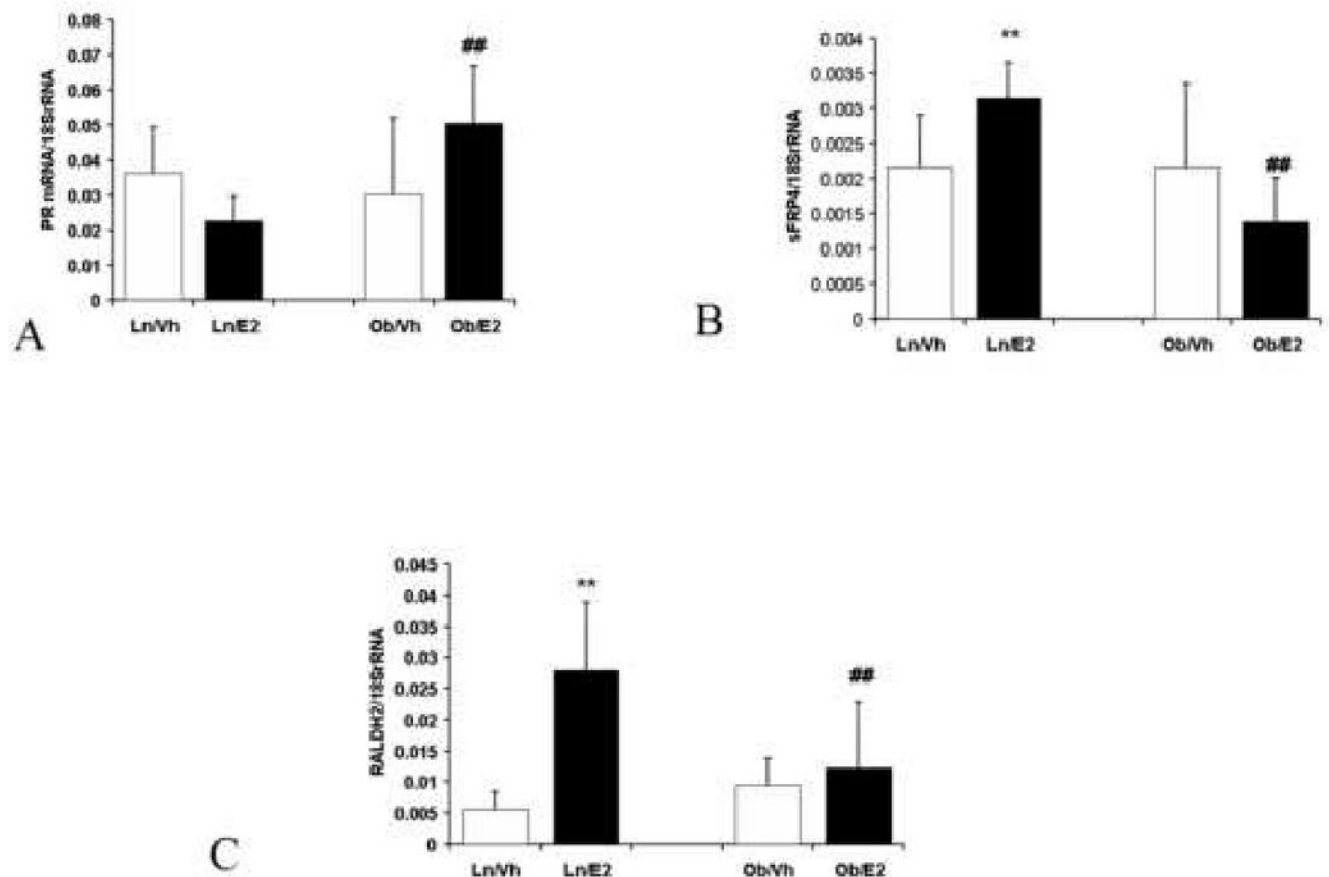
Figure 5. Expression estrogen regulated anti-proliferative gene expression in rat endometrium
Transcript level of progesterone receptor (A), sFRP4 (B), and RALDH2 (C) in the
endometrium of Zucker fa/fa obese and lean rats. 20 ng of endometrial RNA were assayed in
triplicate for RALDH2 transcripts by real-time quantitative RT-PCR. Absolute values were
normalized to and expressed as a percentage of 18S. n =6. **, p < 0.05 compared of lean
estradiol-treated group/Lean Control; ##, p<0.05, obese estradiol-treated group vs. lean
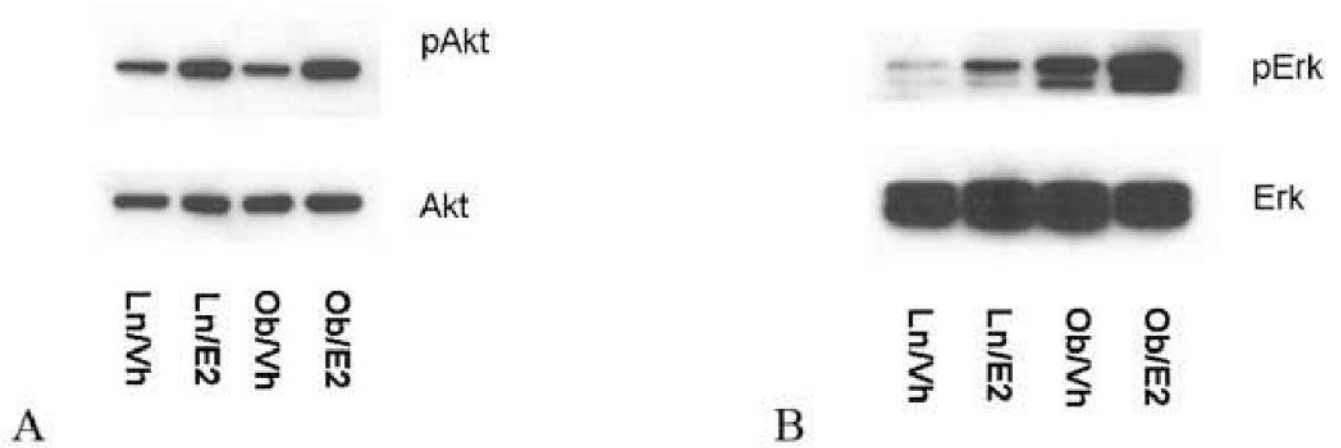estradiol-treated group

Figure 6. Akt and Erk1/2 Phosphorylation in rat uteri
Western blot analysis of Akt (A) and Erk1/2 (B) activiation. 25 μg of uterine protein from each rat was subjected to immunoblot analysis with antibodies specifically against Ser[473] phosphorylated Akt (A) and phosphorylated Erk1/2 MAPK (B) antibody. PVDF membranes were subsequently stripped and reprobed with Akt (A) and Erk (B) antibody. n=6.

Table 1

Probes, primers and details for real-time quantitative RT-PCR assays

Transcript	Taqman primers and probe	Accession Number
18SrRNA	1335(+) GAGGGAGCCTGAGAAACGG	M10098
	1401(-) GTCGGGAGTGGGTAATTTGC	
	1359(+) FAM-TACCACATCCAAGGAAGGCAGCAGG-BHQ1	
	Lowest limit of detection – 2,100 molecules	
	PCR efficiency – 93%	
rRALDH2	327(+) AAGCTTGCAGACTTGGTGGA	NM_053896
	420(-) CGATGTAAAAAGCTTGCAGGA	
	348(+) FAM-CGGGACAGGGCAACTCTTGCAACTA-BHQ1	
	Lowest limit of detection – 160 molecules	
	PCR efficiency – 92%	
rPR	2068(+) TATGCAGGGCATGACAACAC	NM_022847
	2142(-) CCTCTCGCCTGTTGGTTGAG	
	2094(+) FAM-CGACACTTCCAGCTCTTTGCTGACCA-BHQ1	
	Lowest limit of detection – 180 molecules	
	PCR efficiency – 94%	
rsFRP4	578(+) CAAGAAAGGTCCTTTGATGC	NM_053544
	645(-) TTCACCTTTTTGCACTTGC	
	601(+) FAM-CTGTAAACATCTGAGCCCTGATCGG-BHQ1	
	Lowest limit of detection – 200 molecules	
	PCR efficiency – 95%	
rp27Kipl	755(+) GGACCAAATGCCTGACTCG	NM_031762
	818(-) CCTCATCCCTGGACACTGC	
	775(+) FAM-CAGACAGTCCGGCTGGGTTAGCG-BHQ1	
	Lowest limit of detection – 220 molecules	
	PCR efficiency – 95%	
rCyclin A2	39(+) ATGAAGAGGCAGCCAGACATC	NM_053702
	109(-) TCTCCCACTTCAACTAGCCAGT	
	61(+) FAM-CTAACAGTATGCGGGCCATCCTTGTG-BHQ1	
	Lowest limit of detection – 210 molecules	
	PCR efficiency – 95%	
rc-Myc	413(+) TGTATGTGGAGCGGCTTCTC	NM_012603
	476(-) CCTGGTAAGAGGCCAGCTTC	
	435(+)FAM-CCGCTGCCAAACTGGTCTCCG-BHQl	
	Lowest limit of detection – 230 molecules	
	PCR efficiency – 91%	