An IGF1/insulin receptor substrate-1 pathway stimulates a mitotic kinase (cdk1) in the uterine epithelium during the proliferative response to estradiol


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Abstract

Estrogens are potent mitogens for some target organs, such as the uterus, and cancers that develop in this organ might be linked to the proliferative action of these hormones. However, the mechanism by which estrogens influence the cell cycle machinery is not known. We found that a null mutation for the insulin receptor substrate (IRS)-1, a docking protein that is important for IGF1 signaling, compromised hormone-induced mitosis in the uterine epithelium; BrdU incorporation was not affected. This selective effect on mitosis was associated with a reduction in uterine cyclin B-associated kinase activity; cyclin A-associated kinase activity was not changed. The null mutation also reduced the extent of hormone-induced phosphorylation of endogenous uterine histone H1, as determined with phospho-specific antiserum. Uterine epithelial cyclin dependent kinase (cdk)1 was induced in response to hormone, but the level of the kinase protein, as determined by immunoblotting, was noticeably less in the isr1 null mutant than that in the wild-type (WT) mouse, especially around the time of peak mitosis (24 h). Since IRS-1 binds/activates phosphatidylinositol 3-kinase (PI3K), the absence of this docking protein could impair signaling of a known pathway downstream of AKT that stimulates translation of cell cycle components. Indeed, we found that phosphorylation of uterine AKT (Ser473) in isr1 null mutants was less than that in WTs following treatment. Based on earlier studies, it is also possible that an IGF1/IRS-1/PI3K/AKT pathway regulates posttranslational changes in cdk1. This model may provide insights as to how a growth factor pathway can mediate hormone action on cell proliferation.


Introduction

It is well known from laboratory studies of rodents that estradiol (E2) is a potent mitogen for uterine luminal and glandular epithelial cells (Tong & Pollard 2002). This mitotic action extends to other congeners of E2 and may contribute to the increased risk of endometrial carcinoma observed in postmenopausal females provided with estrogen replacement therapy (Gambrell et al. 1983). The proliferative response to E2 occurs naturally during the ovarian cycle and during early pregnancy. Hormone interaction with the uterine estrogen receptor-α (ERα) is apparently essential for this action since ovariectomized ERα knockout (αERKO) mice exhibit negligible uterine cell DNA synthesis or proliferation in response to E2 (Couse et al. 1995). Injection of E2 into the ovariectomized rodent increases both the expression of cell cyclins and the activation of cyclin-dependent kinases (cdk). Transcripts for uterine cyclins D (D1–D3), E, A, and B increase, with each cyclin having a specific temporal pattern following estrogen exposure (Altucci et al. 1997, Tong & Pollard 1999). In mammalian cells, cyclin A is reported to function during S phase, as well as during the G2/M transition, whereas cyclin B controls entry into mitosis (Minshull et al. 1990, Nurse 1990). There is limited knowledge of the local pathways that link activation of the estrogen receptor with the uterine epithelial cell cycle machinery. For over two decades, investigators have considered the notion that hormone-induced growth factors can serve as mediators of cell proliferation. In this regard, experimental evidence is compiling that supports a functional role for the insulin-like growth factor I (IGF1) in estrogen-induced proliferation of uterine epithelial cells. E2 is known to increase the level of IGF1 transcripts in the uterus of some mammals. For instance, IGF1 mRNA increases by 14-fold in the uterus of the ovariectomized rat as early as 6 h after treatment with E2 (Murphy et al. 1987). This elevation of the uterine IGF1 ligand leads to activation of the IGF1 receptor and downstream signaling pathways in the uterus (Richards et al. 1996, 1998, Klotz et al. 2000). Other studies show that the insulin receptor substrate-1 (IRS-1), which functions as
a primary substrate for the insulin and IGF1 receptors, is critical for the mitogenic response of these factors (Waters et al. 1993, Rose et al. 1994, Bruning et al. 1997). The omission of IRS-1 in embryonic fibroblasts by null mutation results in a marked reduction in IGF1-stimulated cell proliferation and phosphatidylinositol 3-kinase (PI3K) activity (Bruning et al. 1997). In situ hybridization studies suggest that the interaction of IGF1 with the uterine epithelial IGF1 receptor occurs through a paracrine-type mechanism, with IGF1 originating in proximal stromal cells (Baker et al. 1996, Cooke et al. 1997). A complex containing the IGF1R, IRS-1, and PI3K occurs in uterine extracts of mice treated with E2 or IGF1 (Richards et al. 1998). Formation of this hormone-induced complex is severely compromised in zERKO mice (Klotz et al. 2000) and also in IGF1/m/m mice (Richards et al. 1998), which have a deficiency in IGF1 (Lembo et al. 1996).

It is well known that IGF1 can stimulate proliferation of a variety of cells in vitro (Lowe 1991). This growth factor was originally referred to as a ‘G1 progression factor’ as an outcome of these earlier studies (Leof et al. 1982, Campisi & Pardee 1984). However, a more recent investigation of cell cycle kinetics in fibroblasts derived from igf1−/− null mice reveals a large increase in the duration of the G2/M phase when compared with that of wild-type (WT) cells (Sell et al. 1994). Moreover, when intact igf1−/− null female mice are treated with E2, the proportion of uterine epithelial cells in the G1 and S phases by 20 h is not different between WT and mutant mice; however, cell number and mitotic figures are markedly reduced in the igf1−/− null mice (Adesanya et al. 1999). This is the direct evidence that IGF1 provides a mitogenic stimulus in the uterus in response to E2 and does so by influencing transit time through G2/M, rather than G1.

The major goal of this study is to identify components of the cell cycle in the uterus that are regulated by the hormone-induced IGF1 signaling pathway. To achieve this, we use the uterine epithelium of hormone-treated ovarioctomized mice as an experimental model (Fagg et al. 1979). This model permits analysis of signaling and cell cycle proteins in the uterine epithelium, while providing the permissive micro-environment for E2 to stimulate cell proliferation (Cooke et al. 2002). Since adult igf1−/− null mutant mice are extremely small and can exhibit a high frequency of neonatal lethality (Liu et al. 1993), is1−/− null mutants (Araki et al. 1994) were selected for most of the experiments. As described above, the uterus of the is1−/− mouse should have a compromised IGF1 signaling pathway. Our initial experiments used igf1−/− mice to establish that this ligand is critical for E2-stimulated association of PI3K (p85) with IRS-1. Following this, we sought to determine whether is1−/− null mutants exhibit compromised mitosis in the uterine epithelium in response to E2, as was previously observed with igf1−/− null mutants (Adesanya et al. 1999). Finally, we attempted to identify what component(s) of the cell cycle might be regulated by the IGF1/IRS-1 pathway. Our findings indicate that the IGF1/IRS-1 pathway contributes to the mitogenic action of E2 by stimulation of cyclin B-associated cdk1 kinase. This finding may have broad implications with regard to the mechanism by which IGF1 provides a stimulus for cell proliferation in various tissues or neoplasias.

Materials and Methods

Mice, treatments, and tissue collection

IRS-1 WT (irs1+/+) and IRS-1 null mutant (irs1−/−) mice on a C57Bl/6×129Sv background were generated in our laboratory from heterozygous IRS-1 breeding pairs obtained from Taconic (Germantown, NY, USA) with the permission of Dr Ronald Kahn (Joslin Diabetes Center, Boston, MA, USA). Null mutants were also generated by breeding is1−/− males with is1−/+ females. The weight of female is1−/− mice was ~70% that of the corresponding WTs. IGF1 WT (igf1+/+) and IGF1 null mutant (igf1−/−) mice were generated from heterozygous IGF1 breeding pairs (MF1×129/Sv hybrids) provided by Dr Argiris Efstratiadis (Columbia University, New York, NY, USA). Tail genomic DNA was screened by PCR to determine the genotype. All of the WT, is1−/−, and igf1−/− mice were ovarioctomized at 8–13 weeks of age. Procedures for surgery, treatments, and euthanasia were conducted in compliance with the guidelines of the NIΕHS, National Institutes of Health Animal Care and Use Committee.

To assess 5-bromo-2-deoxyuridine (Brdu) incorporation, ovarioctomized WT and is1−/− mice (n = 8 per group) were treated with E2 (0.08 μg/g) or vehicle (4% v/v ethanol PBS) s.c. and killed after 18 h; Brdu (Sigma, 0.1 mg/g i.p.) was injected 2 h before euthanasia. For mitosis experiments, mice (n = 4 per group) were killed 22 h after hormone treatment; the anti-mitotic compound demecolcine (Colcemid, Sigma, 5 μg/g i.p.) was injected 2 h before euthanasia. Uteri were excised, and a 5 mm long transverse section in the middle of each horn was taken and immersed in 10% neutral-buffered formalin. Paraffin sections were prepared by standard procedures. Brdu was detected in sections with a rat anti-Brdu monoclonal antibody that was part of a Vector kit (no. PK4004). All mice were treated with hormone no earlier than 14 days after ovarioctomy.

Apoptotic cells were identified in uterine sections with a TACS 2 TdT (TBL) In Situ Apoptosis Detection kit (Trevengen, Gaithersburg, MD, USA) according to the manufacturer’s instructions; negative and positive control slides were included in the kit.

Epithelial cell extracts

Mice were treated with hormone as described above and euthanized at various time points after treatment. The specific intervals (up to 48 h) following treatment for the different experiments are shown in the figures. For the 0 time point, mice were treated with 4% ethanol PBS and immediately killed. Uterine epithelial cells were removed as described.
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previously (Fagg et al. 1979, Tong & Pollard 1999). Briefly, uteri were slit lengthwise and placed in 15 ml tissue culture tubes with chilled extraction buffer (1% Triton X-100, 150 mM NaCl, 1 mM Na3VO4, 1 mM NaF, 50 mM Na2MoO4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 4 μg/ml 4-ami-no-phenylmethylsulfonyl fluoride in 50 mM Tris–HCl, pH 7.4). A volume of 580 and 400 μl buffer was used for each WT and irs1−/− uterus respectively. The uteri were agitated in the presence of seven glass beads (3+0 mm diameter) on a Vortex-Genie mixer (30 s on/30 s off) for a total of 5 min. The resulting cell suspension was drawn off and then disrupted ultrasonically (3X5 s bursts) followed by brief centrifugation. The supernatant was transferred to a microfuge tube and stored at −80°C. Protein concentration was determined with a Pierce BCA assay kit (Pierce Chemical, Rockford, IL, USA) with bovine albumin (2 mg/ml) as a standard.

Western immunoblotting and immunoprecipitation
To remove endogenous IgGs prior to immunoprecipitation, aliquots of supernatants were cleared by incubating with 100 μl washed protein A-Sepharose (CL-4B, Amersham Pharmacia Biotech) for 30 min at 4°C. For immunoprecipitation, the cleared aliquots (300–450 μg protein) were incubated with 5 μg antibody for 2 h at 4°C. After incubation with the antibody, the supernatants were incubated with the protein A-Sepharose for an additional 2 h at 4°C. Antibody/protein A-Sepharose pellets were washed three times with the extraction buffer and subjected to kinase assays or boiled for 5 min in Laemmli sample buffer. After boiling, the precipitates were stored at −20°C. Aliquots of mouse uterine epithelial cell extracts or immunoprecipitates were boiled for an additional 2 min, subjected to SDS-PAGE, and then transferred to polyvinylidene fluoride (Immobilon-P) membrane (Millipore, Bedford, MA, USA). The membrane was blocked with either 5% bovine albumin/Tris–buffered saline (TBS) with 0.1% Tween 20 or 5% nonfat dry milk/PBS and incubated with a dilution of specific antibody. For cdk1, where cytokeratin 18 was used as a loading control, the membrane was cut just above the 37 kDa protein marker. The lower portion (cdk1) was blocked with 1% BSA/TBS-Tween, and the upper portion (cytokeratin 18) was blocked with 5% nonfat dry milk/PBS.

Antibodies (catalog number) to IRS-1 (06-248), p85 (06-195), cdk1 (06-141), and cdk2 (05-596) were purchased from Upstate (Temecula, CA, USA); antibodies to cyclin A (sc-596), cyclin B1 (sc-595), and cytokeratin 18 (sc-28264) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies to AKT (9272) and phospho-AKT (Ser473) (9271) were from Cell Signaling Technology (Beverly, MA, USA).

Each membrane was then incubated with a HRP-conjugated donkey anti-rabbit (NA9340) (Amersham Biosciences) or goat anti-mouse IgG secondary antibody (A3682) from Sigma.

Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Six trials were used to obtain immunoblot of uterine cdk1 and cdk2 for up to 30 h after E2 treatment of WT versus irs1−/− mice. Four trials were used to examine uterine cdk1 levels in both the groups 24 h after E2 treatment with cytokeratin 18 as a loading control. Four trials were also used to analyze p-AKT/AKT in both the groups in a time-course study. In these experiments, different extracts were analyzed for each trial. Densitometric scans of immunoblot bands were made with an Alpha Innotech FluorChem FC2 Imaging System.

Microarray analysis
Fold changes in mouse uterine cdk1 and cdk2 transcripts following treatment with E2 were retrieved from the microarray data offered by Sylvia C Hewitt and Dr Bonnie Deroo (NIEHS, NIH). Details of the experimental design have been published (Hewitt et al. 2003), and the repository for the microarray data is accessible at NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/); accession numbers GSE4664 and GSE4615.

Quantitative real-time PCR
Uteri were obtained from control (0 h; n = 4 for each group) and estrogen-treated mice (24 h; n = 6 for each group). Each uterus removed was immediately placed on ice in a tube containing 1.5 ml RNALater Stabilization Reagent (Ambion, Austin, TX, USA). RNA was extracted with the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Uteri were transferred to the RLT buffer (0.6 ml per uterus) containing β-mercaptoethanol (15 μl/ml), and homogenized with an Omni Model TH-115 hand-held homogenizer (7 mm sawtooth probe with three bursts at full power, each at ten seconds). RNA isolation was followed by a DNA removal step with DNase I. One microgram total RNA was then reverse transcribed using a Gene Amp PCR 29 System 9700 (Applied Biosystems, Foster City, CA, USA) and the SuperScript First-Strand Synthesis System 30 (Invitrogen).

Quantitative real-time PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) on a Bio-Rad iCycler (Bio-Rad). The primers for mouse cdk1 were 5′-GGA-CCT-CAA-GAA-GTA-CCT-GGA-C-3′ (forward) and 5′-CCC-TGG-AGG-ATT-TGG-TGG-AAG-3′ (reverse). The primers for mouse actin were 5′-TGA-CAG-GAT-GCA-GGA-GGA-GA-3′ (forward) and 5′-CGC-TCA-GGA-GGA-GCA-ATG-3′ (reverse). The cDNAs were amplified for 40 cycles (95°C/10 min → 95°C/15 s → 60°C/30 s). Controls included the omission of template or primers. Melt curves of both the amplicons indicated the presence of a single product and the absence of primer–dimer formation. Relative quantitation of cdk1 expression was determined by the comparative Ct method (Pfaffl 2001) with actin as the endogenous reference gene.
Real-time PCR efficiencies for the cdk1 (E=1.97) and actin (E=1.98) were determined from the Q5 Optical System Software (Bio-Rad).

Cyclin-associated kinase assay

Uterine epithelial extracts were prepared as described above and stored at −80 °C. Equal amounts of WT and KO extracts (~400 μg protein) were diluted to 0.5 ml with the extraction buffer. After preclearing the sample with protein A-Sepharose, 5 μg anti-cyclin B (Upstate) or anti-cyclin A (Santa Cruz) were reacted with the sample for 2.5 h. Protein A-Sepharose was added, and the immunoprecipitates collected were washed three times with the extraction buffer and then three times with the cdk1/cdk2 kinase assay buffer (20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2, 25 mM glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol). The kinase reaction was carried out according to the instructions of the cdk1/cdk2 kinase assay kit (Upstate). Kinase activity of the immunoprecipitate was carried out in the assay buffer containing 10 μCi [γ-32P]ATP (3000 Ci/mmol), 90 μM ATP, and 14 mM MgCl2. Histone H1 (0.4 μg/μl) was the exogenous substrate. The mixture was incubated for 10 min at 30 °C. The assay was stopped by placing the reaction tubes on ice and adding gel sample loading buffer. Sample proteins were separated on 4–20% Tris Ready Gels (Bio-Rad) and transblotted to Immobilon-P membrane. Five separate trials, each with different uterine extracts, were used for the kinase assays. Detection was made by autoradiography and phosphoimaging (Storm 860 Molecular Imager).

In situ histone (H1) phosphorylation

Nuclei were isolated from whole uteri following homogenization in a detergent-containing buffer, as described previously (Archer et al. 1991). Nuclei were resuspended in 0.2 M H2SO4 and proteins in the soluble fraction precipitated with 5 volumes of ethanol. Ten micrograms isolated histones were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Immunoblotting procedures were similar to those described above. The membrane was probed with antibodies (1–2 μg/ml) developed in rabbits against mouse H1.3 amino acids 15–27 containing a phosphate at Thr18 (Deterding et al. 2008). A test of the antibody against chromatographically purified H1 isoforms revealed recognition of only H1.3 and H1.4 isoforms in mouse cells; the antibody did not detect an H1.3 peptide phosphorylated at Ser173. Three separate trials, each with different uterine extracts from WT and irs1−/− uteri, were used for the in situ histone phosphorylation experiments.

Statistical analysis

Statistical significance was determined by the Student’s t-test; two-sided P<0.05 was considered statistically significant.

Results

IGF1 is required for E2-induced IRS-1 activation

Since we intended to use the irs1−/− mice to compromise the IGF1R signaling pathway, it was important to ascertain at the outset that the IRS-1 docking protein requires IGF1 for activation of PI3K in response to E2. To demonstrate this, ovariectomized female WT and igf1−/− mice were treated with E2 (6 h), and the immunoprecipitates of IRS-1 from uterine epithelial extracts were evaluated for the PI3K regulatory subunit (p85). As shown in Fig. 1, the immunoprecipitates of uterine IRS-1 from WT mice (lanes 1–4) revealed the expected increased association of p85 as a result of hormone treatment. In contrast, uterine IRS-1 from hormone-treated igf1−/− mice (lanes 5–9) was not associated with p85. These findings indicate that the IGF1 ligand is a required intermediate for estrogens to activate IRS-1 and stimulate binding of p85 to IRS-1 in the uterus. In addition, these data rule out the participation of another IGF1R ligand, e.g. IGF2, that might function in the absence of IGF1 to stimulate the activation of IGF1R and IRS-1 (Osborne et al. 1989).

E2-stimulated uterine epithelial proliferation is compromised in irs1−/− mice

Since IGF1 was critical for E2-induced activation of uterine IRS-1, mice with an IRS-1 null mutation could provide insights as to the importance of an IGF1/IGF1R/IRS-1 pathway in the proliferative response of uterine epithelial cells to E2. In order to determine this, ovariectomized adult irs1−/− and WT (irs1+/-) female mice were treated with E2, and the uterine epithelium was examined for the proportion of cells in the S phase and in the mitosis. The proliferative response of the uterine epithelial cells to E2 in the adult mouse is considered to begin with a drastic shortening of the G1–S

![Figure 1](https://endocrinology-journals.org/207/225-235)
In (A and B), each bar represents the mean of the procedures used are described in Materials and Methods. From that of wild-type (WT) mice. (B) Mitosis in uterine epithelial undergoing mitosis was determined at 22 h after hormone treatment, followed by euthanasia at 22 h. Further details of the procedures used are described in Materials and Methods. In (A and B), each bar represents the mean ± s.d.; n is the number of mice used in each group. For both BrdU uptake (A) and mitosis (B), ~500 epithelial cells were counted in each uterine section. The mitotic index of the KO mice was significantly different from that of WT mice at P<0.005.

transition rate. The epithelial cells exhibit a near-synchronous wave of DNA synthesis beginning about 10 h after hormone treatment; this is followed by mitosis that peaks around 20–24 h. To identify cells in the S phase, BrdU was injected into animals that were previously treated with E2 16 h earlier. Negligible incorporation of BrdU into uterine epithelial cells occurred when WT or mutant mice were injected with vehicle. Incorporation of BrdU was apparent in both the groups of mice, and the proportion of cells that incorporated BrdU (labeling index) in irs1−/− mice (70 ± 13, mean ± s.d.) was not significantly different (P>0.05) from that of WT mice (79 ± 8, mean ± s.d.; Fig. 2A). The fraction of epithelial cells undergoing mitosis was determined at 22 h after hormone treatment. At this time, epithelial cells of the irs1−/− mice had approximately one-sixth the frequency of mitotic figures (1.8 ± 1.2, mean ± s.d.) as that of WTs (10.5 ± 0.3, mean ± s.d.; P<0.005; Fig. 2B). These data suggest that the IRS-1 null mutation affects the rate of transit of uterine epithelial cells through G2/M without impairing recruitment of cells into G1/S in response to E2. The effects obtained with the irs1−/− mice with regard to hormone stimulation of the uterine epithelial cell cycle are similar to that reported previously for igf1−/− mice (Adesanya et al. 1999). In both cases, the null mutation affected the number of cells in mitosis but not the cells in the S phase. This functional overlap indicates that IGF1 and IRS-1 are part of the same signaling pathway that mediates hormonal stimulation of mitosis of cells in the uterine epithelium. The decrease in the number of mitotic figures observed at 22 h after E2 treatment in irs1−/− mice could not be explained by a difference in apoptosis. Evaluation of uterine sections at this time point for detection of fragmented DNA in situ end labeling showed no detectable apoptosis in the epithelium of WT or irs1−/− mutant mice (data not shown).

The IRS-1 null mutation reduces the E2-induced increase in cdk1 (p34) protein but not in mRNA

The significant decrease in hormone-induced mitosis in the uterine epithelium of irs1−/− mice prompted us to consider an effect of the mutation on the major mitotic kinase cdk1. In the mammal, cdk1 kinase is poorly expressed in normal quiescent cells but accumulates during the S phase and peaks during the G2 and M phases of the cell cycle (Kim et al. 1992, Loyer et al. 1994). As shown in Fig. 3A, microarray analysis of WT mouse uterine RNA reveals that the level of cdk1 transcripts increases markedly by 24 h after treatment with E2, whereas the level of cdk2 transcripts was essentially invariate during this period. The influence of E2 on uterine cdk1

Figure 2 BrdU uptake and mitosis in uterine epithelial cells of estradiol-treated wild-type and irs1−/− mice. (A) Mice were treated with estradiol and killed 18 h later. BrdU was injected 2 h prior to euthanasia. Standard methods were used to prepare uterine sections for examination by light microscopy. BrdU was detected in epithelial cells with a rat anti-BrdU monoclonal antibody. The estrogen-stimulated incorporation of BrdU into uterine epithelial cells of irs1−/− (KO) mice was not significantly different (P>0.05) from that of wild-type (WT) mice. (B) Mitosis in uterine epithelial cells was determined by injecting demecolcine into mice 20 h after hormone treatment, followed by euthanasia at 22 h. Further details of the procedures used are described in Materials and Methods. In (A and B), each bar represents the mean ± s.d.; n is the number of mice used in each group. For both BrdU uptake (A) and mitosis (B), ~500 epithelial cells were counted in each uterine section. The mitotic index of the KO mice was significantly different from that of WT mice at P<0.005.

Figure 3 Changes in uterine cdk1 transcripts in wild-type and irs1−/− mice after treatment with estradiol. (A) Total RNA was isolated from whole uteris of C57BL/6 (ovariectomized) mice at the indicated time points after treatment with estradiol. The plots compare the estradiol-induced fold changes in uterine gene expression of cdk1 against that of cdk2, as determined by microarray analyses. Details of the microarray are described in Materials and Methods. The plots were constructed from values given at the website in Hewitt et al. (2003). (B) Total RNA was isolated from whole uteris of ovariectomized wild-type and irs1−/− mice before and after treatment with estradiol. cDNA was prepared from 1 µg RNA and used for quantitative real-time PCR. Quantitation was made by the comparative C t method with actin as the endogenous reference gene. After calculating the C t (cdk1 minus actin), the values (C t) were determined relative to that for untreated wild types. Each bar represents the mean ± s.d.; n is the number of mice used in each group. Note that the mean value for the untreated IRS-1 (KO) was <1.0. Cdk1 transcript level was increased in wild-type (WT) and KO animals 24 h after hormone treatment; the value for KO (24 h) was significantly greater than that of WT (24 h) at P<0.005. Further details of the procedures and primer sequences are described in Materials and Methods.

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mRNA in WT mice was compared with that of ins1−/− by quantitative PCR analyses using the comparative (Ct) method with actin as the reference gene. As shown in Fig. 3B, cdk1 transcripts increased in both the groups by 24 h after estrogen treatment. Interestingly, the level of uterine cdk1 mRNA in the IRS−1 null mutants was significantly greater (P < 0.05) than that of the WT mice at this time point.

To compare the levels of cdk1 protein, the extracts of WT and mutant uterine epithelium were prepared at various time points after hormone treatment and evaluated by immunoblotting. In accordance with the microarray and quantitative PCR data, cdk1 (p34) protein in the quiescent uterine epithelium of ovariectomized mice (time 0), WT or ins1−/−, was below the level of detection (Fig. 4A). When mice were treated with E2, uterine cdk1 increased and could be readily detected by 18 h in WT mice. In contrast, the uterine cdk1 abundance in ins1−/− (KO) mice was generally much less than that observed in WTs at the corresponding intervals after hormone treatment (Fig. 4A). In the four trials that analyzed the extracts from uteri obtained 24 h after estrogen treatment, the levels of cdk1 protein in mutants were significantly less (P < 0.005) than that of WTs: KO (0.41 ± 0.09, mean ± s.d.) versus WT (1.16 ± 0.14, mean ± s.d.); KO/WT = 0.35 (Fig. 4B).

For both WTs and mutants, the level of hormone-induced cdk1 was highest at the 30-h time point, which is after peak mitosis in this model. It is not known whether treatment with E2 alone (‘unopposed’) favors this pattern, which might differ considerably from that of the intact female exposed to sequential cycles of estrogens and progestins. Levels of cdk2 (p33) kinase, which forms a heterodimer with cyclin A or cyclin E, were also examined in the uterine extracts of WT and ins1−/− mice treated with hormone. As shown in Fig. 4 (upper panel), cdk2, in contrast to cdk1, was detected in the extracts before and after hormone treatment. Hormone treatment did not elevate uterine cdk2 protein levels, which is in accordance with the microarray findings (Fig. 3A). The discrepancy observed for cdk1 in WT and ins1−/− mice was not apparent for cdk2.

The stimulation of AKT phosphorylation and cyclin B-associated kinase by E2 is reduced in the ins1−/− mutant

Since hormone-induced mitosis was inhibited by the ins1−/− null mutants, we wanted to resolve whether the mutation compromised mitotic kinase activity. Cdk1 acquires the potential to become a mitotic kinase in mammalian cells when bound to cyclin B (Meijer et al. 1989). It is possible that the observed reduction in cdk1 (protein) in mutants prevented accumulation of enough mitotic kinase for mitosis. Alternatively, the mutation might affect posttranslational changes that regulate cdk1. Activation and inhibition of cdk1 kinase are known to occur by phosphorylation and dephosphorylation of specific sites, and it is likely that some of these modifications are integrated with growth factor pathways. The activation of AKT (Ser473 phosphorylation) can be demonstrated in our experimental model, as shown by the time-course study in Fig. 5. AKT phosphorylation is clearly evident in the extracts from WT uterine epithelium obtained at 12 and 18 h following E2 treatment. Based on densitometric scans obtained at the 12-h time point, the p-AKT/AKT KO/WT ratios at 18 and 24 h after hormone treatment were similar to that reported at 12 h (data not shown). After 24 h, the level of AKT phosphorylation was very weak in both the groups (Fig. 5). These findings suggest that an IRS−1-dependent pathway is utilized for the hormonal activation of uterine epithelial AKT. Other (non-IRS−1) signaling pathways probably account for the phosphorylation of AKT observed in mutant animals.

To determine whether the IRS−1 null mutation can affect mitotic kinase activity, uterine cyclin B-associated kinase...
activity was examined for up to 48 h following treatment of the mice with E₂. The phosphorylation of the histone H1 (exogenous) substrate was clearly evident at 20 h after hormone treatment in WT samples; however, much less kinase activity was observed at 48 h (Fig. 6A). This temporal pattern is in keeping with the importance of cdk1/cyclin B kinase activity for entry into the mitotic phase of the cell cycle (Lew & Kornbluth 1996). The corresponding time-course study with samples from is1−/− uteri revealed much less phosphorylation of histone H1 than that obtained with WT samples. As determined by phosphoimaging, the cyclin B-associated kinase activity at 20 h after E₂ treatment in is1−/− samples (1.25 ± 0.17 × 10⁴, mean ± s.d.) was significantly less (P<0.005) than that of WT samples (3.16 ± 0.22 × 10⁴, mean ± s.d.); KO/WT = 0.38. This effect of the IRS-1 null mutation on hormonal activation of cyclin B-associated kinase activity was not observed with cyclin A-associated kinase activity. As shown in Fig. 6B, the ³²P-labeling of the histone H1 substrate by kinase associated with cyclin A precipitates was comparable in the extracts from WT and is1−/− mice. Immunoblots were made of cyclin B and cyclin A in the extracts obtained after 20 h. The amount of each cyclin appeared comparable in WT and is1−/− samples (Fig. 6B). Therefore, it is unlikely that the disparity between hormone-induced cyclin B-associated kinase activity in is1−/− and WT uteri could be explained by lower cyclin B levels.

Phosphorylation of H1 histone during mitosis entry is considered a downstream event of cdk1/cyclin B (Langan et al. 1989). Therefore, we examined the effect of the IRS-1 null mutation on hormone-induced phosphorylation of endogenous H1 histone with a phospho-specific (pThr18) antiserum to a cognate sequence of phospho (Thr18) mouse histone H1.3. As shown in Fig. 6C, three major bands corresponding to H1 isoforms were detected in the acid extracts of isolated uterine nuclei from WT and is1−/− mice. Twenty hours following hormone treatment, an immunoreactive band was observed in WT extracts that comigrated with the H1.3/H1.4 isoforms. In contrast, hormone-induced phosphorylation of these isoforms isolated from is1−/− mice was much less than that observed with WT mice. This finding suggests that the activation of the IGF1/IRS-1 pathway by E₂ can result in phosphorylation of endogenous histone.

Figure 5 AKT phosphorylation in the uterine epithelium of estradiol-treated wild-type mice and is1−/− (KO) mice. Ovariectomized wild-type (WT) and is1−/− (KO) mice were treated with estradiol, and uterine epithelial extracts were obtained at the time points indicated. Mice at the 0 time point did not receive hormone. Each lane represents analyses of one uterus. Equal amounts of extracted protein (300–400 μg) were used for WT and KO mice in each experiment. AKT was immunoprecipitated from extracts with 5 μg anti-AKT antibody and immunoblots made with anti-phospho-AKT (Ser 473) and anti-AKT antibodies. Further details are described in Materials and Methods. The results shown are representative of four separate trials with different animals. Densitometric scans of the 12-h time point revealed that the uterine p-AKT/AKT in KO mice was significantly less (0.55 ± 0.04, mean ± s.d.) than that of WT mice (1.31 ± 0.16, mean ± s.d.); KO/WT = 0.42.

Figure 6 Cyclin A- and cyclin B- associated kinase activities in the wild-type and is1−/− uterine epithelium. (A) Uterine epithelial extracts were obtained from ovariectomized wild-type (WT) and is1−/− (KO) mice after the indicated interval following treatment with estradiol. Mice at the 0 time point did not receive estradiol. Cyclin B was immunoprecipitated from equal amounts (~400 μg protein) of extracts from WT and KO uteri and analyzed for histone H1 kinase activity. More details of the immunoprecipitation and the kinase assay are described in Materials and Methods. The kinase samples were run on 4–20% Tris Ready Gels and then transblotted to Immobilon-P membrane. Specific quantitation of the ²⁵P-labeled histone in the 20 h WT and KO samples was made by phosphoimaging: n = 5 for each group. Cyclin B-associated kinase activity associated with KO uterine samples (1.25 ± 0.17 × 10⁴, mean ± s.d.) was significantly less (P<0.005) than that of WT samples (3.16 ± 0.22 × 10⁴, mean ± s.d.); KO/WT = 0.38. (B, left) Cyclin A- and cyclin B1-associated kinase activities were measured in uterine epithelial extracts obtained 20 h after hormone treatment. (B, right) Immunoblots were made of cyclin A and cyclin B1 in uterine epithelial extracts obtained from wild-type and mutant mice 20 h after hormone treatment. (C) Histones were enriched from whole uterine nuclear fractions, separated by 16% SDS-PAGE, and transblotted. The upper panel represents staining with Coomassie Blue; in the lower panel, the blot was probed with antisera to mouse phospho (Thr18) histone H1.3/1.4 as described in Materials and Methods. Veh, represents extracts from vehicle-treated, or control, mice. The data in A and B are representative of five separate trials; the data in C are representative of three separate trials. Each trial used extracts from different mice, and each lane represents an extract from a single mouse uterus.
Discussion

The use of the whole animal as an experimental model is important in accurately resolving the qualitative and quantitative responses of hormones. Cell cultures are generally inappropriate for studying estrogen-stimulated proliferation because of the requirement for normal epithelial/stromal interactions (Cooke et al. 1997, 2002). Cell cultures may not only be refractory to hormone action but also impose artifacts as a consequence of immortalization, high rate of cell turnover, or inappropriate activation of signaling pathways. This later aspect is particularly important in the present investigation where we are trying to link an E2-induced turnover, or inappropriate activation of signaling pathways.

PI3K also induces a G2 cell cycle arrest that could be alleviated by activated AKT. Mouse embryonic fibroblasts with an AKT1 null mutation revealed a delayed transition from G2/M to G1 (Kandel et al. 2002). In this study, the reduction in PI3K/AKT activation caused by the IRS-1 null mutation apparently led to a loss of specific stimulation of the G2→M transition. The component(s) of the cell cycle regulated by the hormonally stimulated IRS-1/PI3K/AKT that would specifically influence the mitotic phase is unknown.

In accordance with the reduced rate of E2-induced mitosis in uterine cells of ins-/- mice, there was a marked reduction in cyclin B-associated kinase activity. Other cdks cannot supplant the function of cdk1 as a mitotic kinase in mammalian cells (Nurse 1990, Nigg 1993). On the other hand, cdk1 can associate with A- and B-type cyclins in mammalian cells to phosphorylate histone H1 (Loyer et al. 1994, Swank et al. 1997). Since levels of cyclin A, cdk2, and cyclin A-associated H1 kinase activity were not affected by the IRS-1 null mutation, it is likely that cyclin A predominantly functions in uterine cells as a complex with cdk2. This would favor the notion that IGF1R signaling in these cells targets cdk1, and not other cdks, such as cdk2. The level of cdk1 in the IRS-1 null mutants prior to mitosis was less than that in WT, which could delay the formation of the threshold concentration of the mitotic kinase (heterodimer). Since the lower levels of cdk1 protein could not be explained by a corresponding decrease in transcript, altered signaling in the mutant might have reduced translation or half-life of cdk1. Earlier studies showed that IRS-1 and PI3K were required for insulin to stimulate eIF-4E/4E-BP-1 phosphorylation and translation in cultured cells (Mendez et al. 1996). Using phospho-specific antisera, we demonstrated that the translation effectors mechanistic target of rapamycin, tuberin, 4E-BP1, eIF-4E, p70S6 kinase, and glycogen synthase kinase 3 are activated in response to E2; however, we could not demonstrate an effect of the IRS-1 null mutation on specific phosphorylation of these translational components (data not shown). To our knowledge, this specific effect of the IGF1R signaling on cdk1 protein levels has not been previously reported. One study did suggest that translational control of cdk1 (p34cdk1) was associated with meiotic competence of mouse oocytes; incompetent oocytes have abundant cdk1 mRNA but low levels of cdk1 protein (deVantery et al. 1997). Our experimental model may have allowed us to identify changes in cell cycle components that do not occur or are obscured by most cells in culture. With many immortalized cell lines, for instance, the cdk1 protein is expressed at all stages of the cell cycle (McGowan et al. 1990, Welch & Wang 1992, Draetta & Beach 1998). However, there are aspects of cdk1 transcripts in mammalian cell cultures that overlap with those observed in animal studies. For instance, cdk1 transcription in HeLa cells is elevated in the S and G2 phases and low in the G1 phase of the cell cycle, based on nuclear run-on assays (Dalton 1992). Likewise, other studies with HeLa cells revealed that the abundance of cdk1 mRNA and rate of synthesis of cdk1 protein (p34) increase dramatically as the cells pass through S/G2 (Welch & Wang 1992).
Since the KO/WT for p-AKT/AKT was similar at different times (12, 18, and 24 h) after hormone treatment, it is likely that the IRS-1 null mutation does not affect the rate of p-AKT dephosphorylation at Ser473. During the period when uterine AKT phosphorylation was elevated in WT and *ins1*−/− mice, AKT was essentially unchanged from that of untreated mice; the increased level of AKT observed after 24 h following treatment with E2 is in keeping with the known increase in AKT transcripts in response to this hormone (Hewitt et al. 2003). It was reasonable to expect in our model that the IRS-1 null mutation would cause a pronounced reduction in AKT activation. Previous reports suggest that enzymes that regulate phosphorylation and activity of the mitotic kinase are proximal targets of AKT. For instance, phosphorylation of the Wee family of kinases, inhibits the kinase activity of cdk1 (Mueller et al. 2005). A study with starfish oocytes, it was shown that AKT, acting downstream from PI3K, phosphorylates Myt1 and downregulates its activity, which favors the meiotic G2/M phase transition (Okumura et al. 2002). A related study showed that AKT can phosphorylate Wee 1 in 293T or HeLa cells, which promotes cytoplasmic localization at the kinase (by binding to 14-3-3σ), and G2/M cell progression (Katayama et al. 2005). Thus, it is possible that a deficient PI3K/AKT signaling pathway in the IRS-1 null mutants increases phosphorylation at Thr14/Tyr15 of cdk1 and thereby compromises its kinase activity. Alternatively, AKT might also be activating cdc25C, which dephosphorylates Thr14/Tyr15 of cdk1. Inhibition of PI3K by LY294002 in HeLa cells delays hyperphosphorylation of cdc25C and cells entering mitosis (Dangi et al. 2003). However, it is still not known whether a PI3K/AKT pathway is activating cdc25C, directly or indirectly. In another study, wortmannin, a potent inhibitor of PI3K, partially inhibited cyclin B translation and inhibited dephosphorylation of the tyrosine (inhibitory) site of cdk1 of sea urchin oocytes (Salau¨n et al. 2002). In the later study, the effects of wortmannin on cyclin B synthesis (triggered by fertilization) were also correlated with the effects of this compound on cap-dependent translation machinery, which included 4E-BP/eIF-4E dissociation. AKT facilitates the G2/M phase transition of mouse PC12 neuronal cells (Lee et al. 2005). Stable expression of a dominant negative form of AKT suppressed expression of both cdk1 and cyclin B1 at the mRNA and protein levels without affecting the cyclin A or cdk2 protein levels. A constitutively active form of AKT (myristoylated AKT) elevated cyclin B and cdk1 mRNA and protein levels and could override cell growth arrest at G2/M (induced by etoposide).

The present finding that the hormone-induced phosphorylation of an endogenous histone H1 isoform in WTs clearly exceeded that of *ins1*−/− mice is in accordance with the observed decrease of cyclin B-associated kinase activity in the mutant animals. The purified cdk1/cyclin B heterodimer phosphorylates specific sites on histone H1 in vitro (Swank et al. 1997). Earlier studies had also provided evidence to show that the cdk5s are the major in vitro kinases for H1 at cell cycle transitions with cdk1 implicated as the G2 kinase for H1 (Langan et al. 1989). The phosphorylation of histone H1 in vivo by a cdk is considered to destabilize H1–chromatin interactions and, thereby, facilitate a more open chromatin structure (Contrera et al. 2003). Thus, by regulating cyclin B–associated kinase activity an IGF1R/IRS-1/PI3K–stimulated signaling cascade mediates hormonal effects on the state of chromatin condensation. A query to consider for postmenopausal females receiving prolonged exposure to estrogens is whether the perturbation of chromatin structure by this signaling pathway facilitates genomic instability and increased risk of endometrial cancer (Enders & Maude 2006).

Our cumulative findings support that the stimulation of uterine epithelial cell proliferation by E2 is mediated by an IGF1R/IRS-1/PI3K/AKT pathway, and that this pathway targets the mitotic kinase cdk1/cyclin B. A specific goal of future work should determine whether impairment of AKT activity by the IRS-1 null mutation affects the activity of enzymes or other proteins that regulate posttranslational changes or catalytic activity of cdk1. Other cell populations might have mechanisms to regulate mitotic kinase activity that are similar to or overlap with those observed in this study. In a broad sense, our experimental findings are probably part of a more general phenomenon, where individual growth factors exert stage-specific regulation of the cell cycle by activating or suppressing specific cyclin-associated kinases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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