Expression of LH receptor in nonpregnant mouse endometrium: LH induction of 3β-HSD and *de novo* synthesis of progesterone

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Abstract

In mouse uterus, at the late diestrus stage LH binding sites have previously been described. The aim of our study was to confirm the existence of *LH* receptor (*Lhr* (*Lhcgr*)) mRNA and its protein in mouse endometrium. Endometrium at all stages of the estrous cycle contained *Lhr* mRNA, essentially identical to that found in mouse ovary. Endometrium also contained a 72 kDa immunoreactive receptor protein that bound to mouse anti-LHR antibody in western blot. Both receptor mRNA and protein were maximally expressed in the endometrium at metestrus and LH caused a significant increase in their expression levels. Endometrium also contained 3β-hydroxy steroid dehydrogenase (*3β-hsd*) mRNA and 3β-HSD protein. LH addition elevated their expression and activity as evident from increased conversion of labeled pregnenolone to progesterone (P_4) and *de novo* P_4 synthesis. LH-induced endometrial P_4 synthesis is mediated through expression of steroidogenic acute regulatory (*Star*) gene. Results demonstrated that LH-induced P_4 synthesis in endometrium is possibly mediated through the cAMP pathway. Involvement of a MAPK pathway was also evident. Gonadotropin-stimulated endometrial P_4 synthesis was markedly attenuated by an antagonist of MEK1/2, PD98059. LH-stimulated MEK1/2-dependent phosphorylation of ERK1/2 in a concentration- and time-dependant manner in cultured endometrial tissues. Moreover, involvement of cAMP in LH-stimulated activation of ERK1/2 was also evident. It is therefore possible that the major signaling pathways regulating endometrial steroidogenesis in mouse, including the adenylate cyclase and MAP kinase pathways, converge at a point distal to activation of protein kinase A and ERK1/2.

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Introduction

The receptor for LH and human chorionic gonadotropin (HCG) has been classically described as a receptor in the gonads and upon ligand binding regulates the synthesis of steroids that control reproductive processes. Contrary to this conventional belief, numerous studies demonstrated that the LH receptor (LHR)/HCG receptor is also present in a variety of non-gonadal tissues, particularly in the female reproductive tract (Bonnamy *et al.* 1990, Reshef *et al.* 1990, Lei *et al.* 1993, Mukherjee *et al.* 1994, Shemesh *et al.* 1997, Ziecik *et al.* 2005). Activation of this receptor *in vivo* and *in vitro* resulted in a wide variety of responses, which can be attributed to initiation and maintenance of pregnancy (Banaszak *et al.* 2000, Srisuparp *et al.* 2003, Lin *et al.* 2005, Ziecik *et al.* 2005).

Despite the evidence regarding the presence of the LHR/HCG receptor in the endometrium, a few studies indicated that gonadal LHR does not appear to be present in human endometrial tissues. Presence of a portion of the transmembrane part of the receptor molecule suggests that human endometrium may express a truncated or variant form of the receptor (Stewart *et al.* 1999). Reports are even available that HCG does not directly affect *in vitro* decidualization of human endometrial stromal cells (Kasahara *et al.* 2001). It has also been shown that although LH and FSH evoke a growth response in different endometrial cell lines, their stimulation does not appear to be via the classical gonadotropin receptor pathway, and all these together indicate a contradictory role of LHR in the endometrium (Davies *et al.* 2000, Srisuparp *et al.* 2003).

Although the presence of uterine LHR has been demonstrated in many species, information is scanty on its existence and regulation by LH in rodent endometrium at different days of the estrous cycle. Moreover, although different paracrine functions of LH in the endometrium like prostaglandin biosynthesis (Srisuparp *et al.* 2003) or modulation of decidualization (Banaszak *et al.* 2000, Licht *et al.* 2002) have been proposed, LH-induced steroid biosynthesis in nonpregnant mouse endometrium is little understood.

This study aimed to investigate 1) whether *Lhr* (*Lhcgr*) mRNA and receptor are expressed in mouse endometrium at different stages of the estrous cycle and LH can regulate their expression; 2) whether 3β-hydroxy steroid dehydrogenase (*3β-hsd*) mRNA and 3β-HSD protein are expressed in such

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tissue and LH can regulate their expression leading to *de novo* synthesis of progesterone (P_4); and finally 3) the possible signaling pathway(s) of LH-induced *de novo* P_4 synthesis in mouse endometrium.

Materials and Methods

Chemicals

DMEM, bovine calf serum, dibutyryl cAMP (dbcAMP), forskolin (FK), and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma Chemicals. Ovine LH (oLH) was a gift from Dr A F Parlow (NIADDK; lot. no. oLH-26, AFP 5551B). Adenylate cyclase inhibitor, SQ22536 (RBI, Natrick, MA, USA), was a gift from Dr Sibsankar Roy, Molecular Endocrinology Laboratory, Indian Institute of Chemical Biology. Mouse monoclonal LHR antibody was a generous gift from Dr Nafis Rahman, University of Turku, Turku, Finland. MEK inhibitor, PD98059, mouse monoclonal anti-phospho ERK1/2 antibody (p-ERK), mouse monoclonal IgG1 β-actin primary antibody (sc-47778), and the secondary antibody goat anti-96 mouse IgG2α were purchased from Santa Cruz Biotechnology, Mouse monoclonal p-ERK (p-E-4) was recommended for detection of ERK1 phosphorylation at tyrosine-204 and corresponding ERK2 phosphorylation of multiple species. [4,7-^{3}H]pregnenolone (sp. activity 11 000 mCi/mmol) and [1,2,6,7-^{3}H]P_4 (sp. activity 6800 mCi/mmol) were purchased from Amersham International, [^{14}C]leucine (0·1 mCi, sp. activity 210 mCi/mM) was procured from Board of Radiation and Isotope Technology, Mumbai, India. Highly purified P_4 antiserum was donated by Prof. Gordon Niswender (Colorado State University, Fort Collins, CO, USA). Cross-reactivities of various steroids with P_4 antibody were checked before use. Total RNA isolation (TRI) reagent was purchased from Ambion, Inc., Foster City, CA, USA. Smart PCR cDNA synthesis kit was purchased from Clontech. Revert-Aid M-MulV RT reverse transcriptase and deoxy-NTPs were purchased from MBI Fermentas, USA, and Taq DNA polymerase from Invitrogen. DNase was procured from Message Clean DNase I, GenHunter; oligo-dT oligodeoxynucleotide primers (T12–18) and random hexamer primers were purchased from Gibco-BRL; and SYBR Green I Master Mix was from Applied Biosystems, Inc. The stock solution of SQ22536 and PD98059 was prepared in dimethyl sulfoxide so that the final concentration of the solvent in the incubation medium was $<0·1\%$.

Animals

Virgin female mice (7–9 weeks, 20–25 g body weight) were purchased from Animal Division, Indian Institute of Chemical Biology, Kolkata, India, housed at 22 °C under controlled lighting (12 h light:12 h darkness cycle) and provided with food and water *ad libitum*. Vaginal smears were examined for two consecutive cycles and only the normally cycling mice were used. Ethical approval was obtained from the Animal Ethics Committee, University of Kalyani under Committee for the Purpose of Control and Supervision of Experiments on Animals (India).

Tissue preparation and incubation

Mice were killed between 0800 and 0900 h by cervical dislocation. Uterine horns, ovary, kidney, and liver tissues were immediately dissected out, flushed with 0·9% NaCl, and kept in ice-cold DMEM. Uterine horns were slit lengthwise and total endometrial tissues were scrapped with a fine scalpel. Endometrial tissues containing epithelial and stromal cells were then minced under ice and used in culture. A portion of aliquots (40–50 mg of each) of minced endometrial tissues from proestrus, estrus, metestrus, and diestrus mice and minced ovary, kidney, and liver tissues (40–50 mg of each) from metestrus mice were kept at −70 °C for RT-PCR, real-time PCR, and western blot analyses. Subsequently, aliquots (40–50 mg each) of only endometrial tissues from all the stages were placed in a 24-well culture plate (Tarson, Kolkata, India) that contained serum-free DMEM (SF-DMEM) with streptomycin (100 μg/ml) and penicillin (100 IU/ml) at pH 7·2 for the following treatments: a) incubated for 0–8 h with or without LH (100 ng/ml; optimal over the range of 25–200 ng/ml) for RT and real-time PCR of LHR mRNA; RT-PCR of mRNA for steroid acute regulatory protein (*Star*) and *3β-Hsd*; and western blot analyses of LHR protein; b) incubated for 8 h with or without LH (100 ng/ml) in the presence of [^{3}H]pregnenolone (P5) and NAD for determination of 3β-HSD activity; c) incubated for 12 h with increasing concentrations of LH (0–200 ng/ml), or for 2–12 h with or without LH (100 ng/ml) or incubated for 12 h in the presence or absence of modulators of adenylate cyclase (FK) and protein kinase A (PKA; dbcAMP), inhibitor of adenylate cyclase (SQ22536), and inhibitor of MEK1/2, PD98059 for the estimation of P_4 in media; d) incubated with increasing concentration of LH (0–200 ng/ml) for 120 min, or varying time periods (0–120 min), with LH (100 ng/ml) in the presence or absence of PD98059; and e) incubated for 120 min with LH (100 ng/ml), FK (10 μM), dbcAMP (10 mM), or SQ22536 (1·0 mM) in the presence or absence of PD98059 (5·0 μM) for determination of ERK1/2 phosphorylation and total ERK1/2 protein.

In a separate experiment, mice (total number 145) at metestrus stage were ovariectomized (OVX) in the morning (0600 h) and kept for 7 days before the experiment. OVX mice were then given three injections (i.p.) of either LH (500 ng/100 g body weight) or 0·9% saline as vehicle (control) or P_4 (i.m. 4 mg/100 g body weight) or with oil as vehicle on alternate days for 3 days. On day 8, all the mice, including a group of intact mice at metestrus injected with 0·9% saline, were killed; uteri were removed, weighed, and processed for histological preparation. A portion of tissues

from all the control and treatment groups were separately subjected to *in vitro* incubation in the presence of [^{14}C]leucine (0·1 mCi. sp. activity 210 mCi/mmol) with other cold amino acids for 6 h for monitoring of protein synthesis following the procedure described previously (Mukherjee *et al.* 1994).

For RT- and real-time PCR analyses, tissues were rapidly immersed in TRI reagent and stored at −80 °C, and for western blotting, tissues were homogenized in cell lysis buffer (50 mM Tris–HCl buffer pH 8·0, containing 150 mM NaCl, 100 mM EDTA, 0·02% NaN_3, 1% Triton X-100, 100 μg/ml phenylmethylsulphonyl fluoride, and 1 μg/ml aprotinin). 3β-HSD enzyme activity was measured following the procedure described previously (Debnath *et al.* 2000). In brief, endometrial tissues (50 mg each) were incubated with or without LH (100 ng/ml) or trilostane (a specific inhibitor of 3β-HSD) in the presence of [^{3}H]pregnenolone (2×10^6 c.p.m., 138 pmol) and NAD (300 mM) in 1 ml SF-DMEM with constant shaking for 8 h. Inhibitor was added 1 h before LH addition. To estimate media P_4 content after incubations, aliquots of 100 μl each were taken and subjected to steroid extraction.

Isolation of RNA and reverse transcriptase PCR

Total RNA from tissues was extracted using TRI reagent solution following the manufacturer's instruction and the single-step method of Chomczynski & Sacchi (1987) as described previously (Paul *et al.* 2010). RNA purity was determined by A260/A280 ratio and quality was checked by agarose-formaldehyde gel electrophoresis. cDNA from 1 μg RNA was synthesized using the Smart-PCR cDNA synthesis kit following the manufacturer's instruction. Gene-specific oligonucleotide primers were developed using Primer 3beta Software (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA, USA) from the published sequence of the mice genome. The primers of the respective genes with the accession number and their amplified segments are listed in Table 1. Details of RT-PCR have been described previously (Paul *et al.* 2008, 2010). Briefly, from the prepared cDNA, 2 μl were used as template for RT-PCR with gene-specific primers and relative expression was observed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer. A 50 μl PCR volume was made by adding 2·5 U Taq DNA polymerase to a PCR mixture that contained 1×reaction buffer, 200 μM of each deoxy-NTPs, and 20 pmol of each primer. The PCR was performed for 35 cycles of denaturation at 94 °C for 30 s (5 min in the first cycle), annealing at a specific temperature for each set of primers for 30 s, and extension at 72 °C for 30 s (10 min in the last cycle; Applied Biosystems, Inc.). After amplification, the samples were separated on a 1% agarose gel, stained with ethidium bromide, and photographed under u.v. light. The 202 bp fragment of *Lhr* mRNA, 314 bp fragment of *3β-hsd* mRNA, and 186 bp fragment of *Star* mRNA were extracted and sequenced with the upper primer using an automated sequencer (ABI PRISM 3730x1 genetic analyser, Applied Biosystems, Inc.). To obtain full-length LHR sequence in mouse uterus, PCR-amplified cDNA was subjected to Universal Amplification using the 3′- and 5′-RACE system of Invitrogen. Universal Amplification Primer (sequence 5′-(CUA)4 GGCCA CGCGT CGACT AGTAC-3′) contains three restriction endonuclease sites (SalI, MluI, and Spe I). The PCR product (202 bp) was sequenced and subjected to NCBI homology BLAST with the available sequence database of the mouse ovarian LHR.

Real-time PCR

Changes in *Lhr* mRNA expression were confirmed and quantified using CFX96 Real Time PCR detection system (Bio-Rad Laboratories, Inc.) with SYBR Green Master Mix (Applied Biosystems, Inc.). Total RNA extracted from tissues for RT-PCR was used to generate cDNA by RT and the gene-specific primer used for RT-PCR analysis was used for real-time PCR detection of the gene of interest (Table 1). The PCR conditions were set up as follows: after incubation at 50 °C for 2 min and denaturing at 96 °C for 10 min, 45 cycles of 95 °C for 30 s and 62 °C for 1 min were performed. To quantify transcripts of the *Lhr* gene precisely, β-actin transcripts were monitored as the quantitative control and the sample was normalized on the basis of its β-actin transcript content. The primer probe mixture for β-actin was purchased from Perkin–Elmer Applied Biosystems and the method of PCR was followed according to the manufacturer's protocol. The relative difference between each cycle and treatment group was determined using the $\Delta\Delta C_T$ method as outlined in the Applied Biosystems protocol, and the results were presented as fold increases. All PCRs were performed in triplicate and the results were averaged.

Table 1 Primers used in semiquantitative RT-PCR and real-time PCR 62×55 mm (300×300 dpi)

Gene product	**Forward primer** (5′→3′)	**Reverse primer** (5′→3′)	**Size of amplicon** (bp)
Lhr (M81310)	CTGAAAACTCTGCCCTCCAG	CAGTGGCTGGGATTACGATT	202
3β-hsd (NM_008293)	ATGGCCACGAGGAACAGAATCATG	CTGTCCTTGGATGCTTGTAGACTTC	314
Star (NM_011485.4)	CAGTCCCGGGTGGATGGGTCA	TCCCCGTTCTCCTGCTGGCT	186
Gapdh (NM_001001303)	GTACAAGGAGAACCAAGCAACGAC	GTGCCGTCTTTCATTACACAGGAC	450
β-Actin	ATATCGCTGCGCTGGTCGTC	AGGATGGCGTGAGGGAGAGC	517

Electrophoresis and immunoblotting

For LHR protein analysis, minced endometrial tissues of proestrus to diestrus stage mice were pre-incubated in DMEM for 2 h followed by incubation in SF-DMEM in the presence or absence of LH (100 ng/ml) for different time intervals. For determination of ERK1/2 phosphorylation, minced endometrium at metestrus was pre-incubated in DMEM for 2 h followed by incubation in SF-DMEM. SF-DMEM medium was replaced twice to reduce basal ERK1/2 phosphorylation. Finally, tissues with SF-DMEM media were incubated with increasing concentrations of LH or varying times containing various stimulators and inhibitors. After incubation, tissues both for LHR protein analysis and determination of ERK1/2 phosphorylation, were separately lysed with lysis buffer. Lysate, after keeping for 15 min on ice, was sonicated and centrifuged at 12 000 ***g*** for 5 min and the protein content of the supernatant was determined (Lowry *et al.* 1951). For immunoblotting, 20 μg total protein were electrophoresed through a 10% SDS–PAGE and were transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were first blocked for 1 h in blocking solution (Tris-buffered saline with 0·1% Tween 20 and 5% non-fat milk) followed by incubation with primary antibodies overnight at 4 °C. Mouse monoclonal anti-LHR antibody for mouse LHR, mouse monoclonal anti-phospho ERK1/2 antibody P-ERK (E4), and mouse monoclonal β-actin antibody were used at 1:2000 dilution. Membrane was also probed with mouse monoclonal antibodies for total ERK1/2 (to confirm equal protein loading) at a dilution of 1:1000. Bound primary antibodies were visualized using corresponding secondary antibodies (goat anti-mouse IgG; 1:1500 dilutions), which were tagged with alkaline phosphatase and were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Extraction, identification, and quantification of steroids

The methods of extraction and estimation of labeled steroids for determination of 3β-HSD activity and estimation of P_4 from incubation media by RIA were similar to those described previously (Debnath *et al.* 2000). The sensitivity of the RIA was 12 pg/incubation and the intra- and interassay coefficients of variation were 7 and 9% respectively.

Statistical analysis

Data from triplicate tissue incubations from a single-donor mouse showed a similar tendency and a mean of them was considered as one experiment. Data were expressed as mean ± S.E.M. of three or four such experiments as stated in the figure legends. Following tests for normality and homogeneity, the significance of treatment effects was determined by one-way ANOVA within and across different effectors. Individual comparisons between treatments were made using Bonferroni's multiple comparison tests using SPSS (Chicago, IL, USA) with a significance level of $P<0{\cdot}05$.

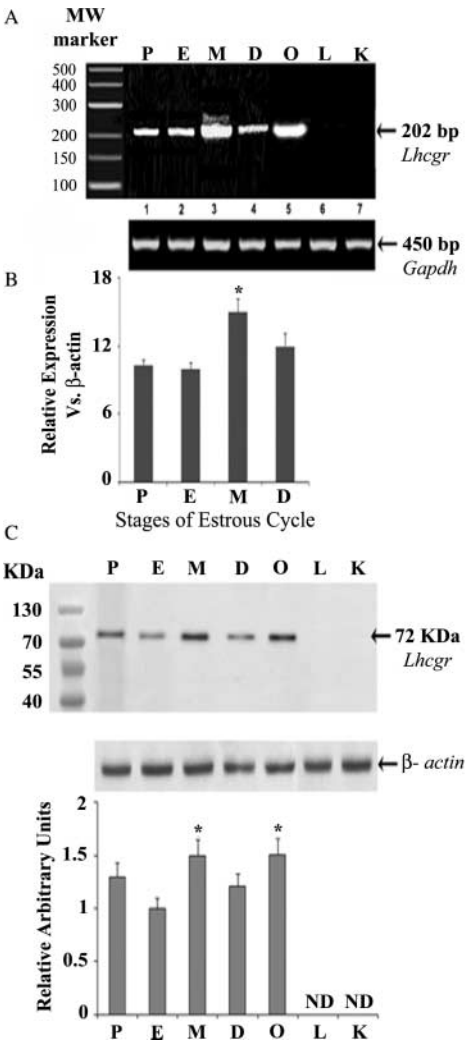


Figure 1 RT-PCR (A) and real-time PCR (B) amplification of mouse endometrium *Lhr* mRNA. RT-PCR was performed using 1 μg RNA from endometrium of proestrus (P), estrus (E), metestrus (M), and diestrus (D) (lanes 1, 2, 3, and 4) stage mouse. Lanes 5, 6, and 7 represent the positive control (O, ovary at metestrus) and the negative controls (L, liver; K, kidney) respectively. PCR amplification was performed as described in the Materials and Methods section. *Gapdh* mRNA was used as loading control for RT-PCR and β-actin was used to normalize the abundance of *Lhr* mRNA in real-time PCR. Expression of LHR protein in mouse endometrium (C). Tissue extracts were prepared and analyzed as described in the Materials and Methods section. Twenty micrograms of protein of soluble extract of endometrium at various stages of the cycle (P, E, M, and D) or ovarian (O), liver (L), and kidney (K) tissues from metestrus were separated on SDS–PAGE and electroblotted. The binding of mouse anti-LHR antibody (1: 2000 dilutions) was then used in western blot to determine the presence of LHR protein. β-Actin was used as a loading control. The pixel densities of the bands (lower panel, C) were quantified with ImageJ Software (National Institute of Health (NIH)) and have been represented in the bar diagram as relative arbitrary units considering the control value as 1. (A and C) are representative of four independent experiments, each performed in triplicate. Each bar in (B) represents the mean normalized expression (±S.E.M.) of three independent tissue pools (three mice per pool). $*P<0{\cdot}05$.

Results

Lhr *mRNA in mouse endometrium*

A sequence homology search of the 2103 bp PCR fragment obtained by 5′- and 3′-RACE revealed that mouse endometrial LHR is 100% identical with mouse (*Mus musculus*) ovarian *Lh/Cgr* mRNA (data not shown).

RT-PCR demonstrated that *Lhr* mRNA is expressed in the fresh endometrial tissues obtained from proestrus to diestrus and ovarian tissue (used as positive control) from metestrus stage mice (Fig. 1A). Absence of *Lhr* mRNA expression in kidney and liver tissues (used as negative controls) from metestrus mice indicates that not all tissues of female mice express *Lhr* mRNA (Fig. 1A). Both in endometrial and ovarian tissues, occurrence of a single band for *Lhr* mRNA indicate that it is a pure product. *Lhr* mRNA expression level in the endometrium from metestrus stage was comparatively higher than those of other stages. To demonstrate that the presence of *Lhr* mRNA in endometrial or ovarian tissues and its absence in liver and kidney was specific, *Gapdh* mRNA was used as loading control. RT-PCR amplification using primers described in Table 1 demonstrated that a specific 450 bp band was produced from GAPDH cDNA (Fig. 1A; lower panel). The 450 bp fragments were also found in liver and kidney preparation even though they were negative for the 202 bp band that corresponded to the *Lhr* mRNA. The nucleotide sequence of 202 bp fragment obtained from mouse endometrial LHR displayed 99% homology with the comparable region of *M. musculus Lhcgr* mRNA reported by Gudermann *et al.* (1992; Fig. 2).

Endometrial *Lhr* mRNA was quantitated by real-time PCR and mouse β-actin mRNA was used as an internal control to normalize abundance of *Lhr* mRNA. The size of the β-actin amplicon is 517 bp. Figure 1B demonstrates that *Lhr* mRNA is expressed in endometrium of all the four stages and a comparatively higher level ($P<0{\cdot}05$) was observed in tissues from metestrus than from other stages (Fig. 1B).

LHR protein in mouse endometrium

In order to address the next obvious question about the translation of the transcribed mRNA, western blot was performed. As shown in Fig. 1C, a 72 kDa protein was identified in endometrial tissues of all the four stages in mice with mouse anti-LHR antibody and that corresponded fully with the protein isolated from ovarian tissues at metestrus stage that was used as positive control. Figure 1C also shows that a comparatively stronger signal for 72 kDa LHR was present in endometrial tissues from metestrus ($P<0{\cdot}05$) than from other stages. Tissue negative for LHR protein in extracts of fresh liver and kidney showed negative signal (Fig. 1C).

Effect of Lh on Lhr *mRNA and protein expression*

Mouse endometrial tissues from all the four stages in mice were incubated with LH for different time intervals (0–8 h).

Figure 2 Automated nucleotide sequencing and homology analysis between *Mus musculus Lhcgr* mRNA (accession no. NM_013582.2) from nucleotide 1675 to 1877 and the PCR products were obtained using the upper primer described in the Materials and Methods section. A homology of 99% was found between the *M. musculus* LH/choriogonadotropin receptor and the PCR product for a 202 bp fragment, suggesting that our amplified cDNA is complementary to *Lhcgr* mRNA.

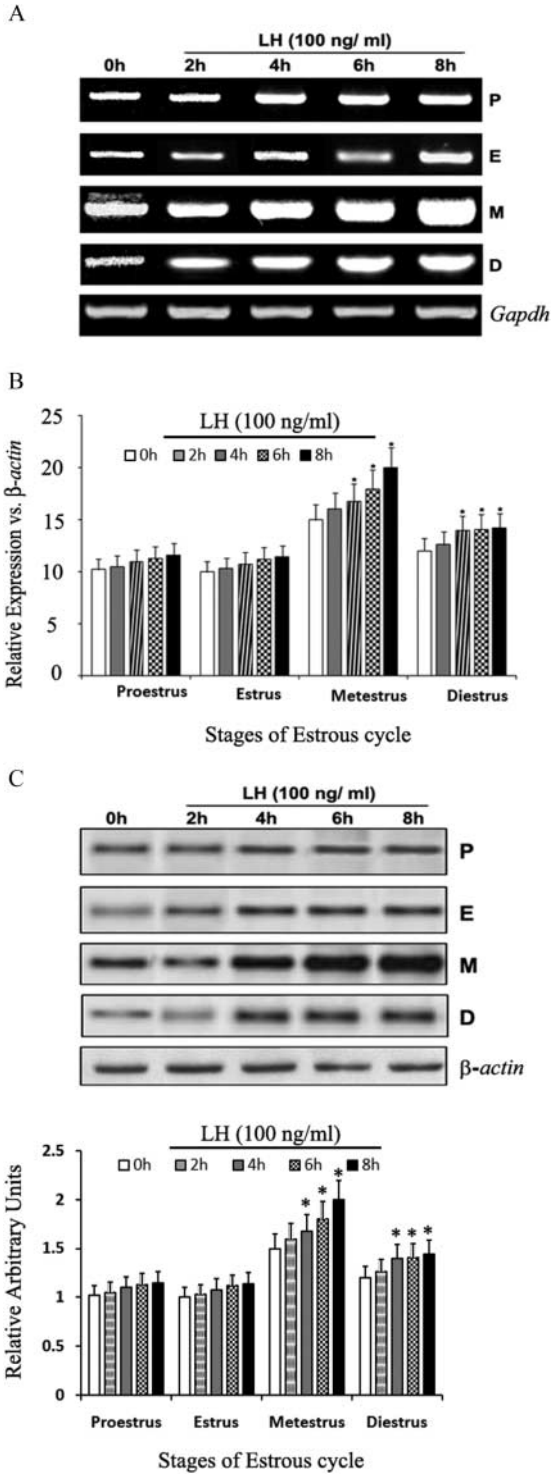
RT-PCR demonstrated that addition of LH (100 ng/ml) to the incubations caused very little increase in the expression levels of *Lhr* mRNA in proestrus and estrus tissues from 2 to 8 h compared with 0 h for the control (Fig. 3A). Tissues from metestrus, however, registered very high expression levels after LH addition, with increasing time and maximum expression recorded at 6 and 8 h. In diestrus, a prominent increase in the expression levels was also noticed up to 4 h after LH treatment with no further increase thereafter (Fig. 3A).

In real-time PCR, a very little increase in the expression of *Lhr* mRNA level was noticed in tissues from proestrus and estrus up to 8-h incubation with LH (100 ng/ml) compared with 0 h with control (Fig. 3B). A gradual and significant increase ($P<0{\cdot}05$) in the expression levels was obtained in metestrus tissues incubated with LH for increasing times with a maximum at 8 h (Fig. 3B). Tissues from diestrus stage mice also showed a significant increase in the expression levels up to 4 h with no further increase thereafter (Fig. 3B).

Western blot analyses of LHR protein showed no remarkable change in the signal for the 72 kDa protein up to 8 h of LH treatment in tissues obtained from proestrus and estrus stage mice (Fig. 3C). In metestrus tissue, however, LH addition to the incubations caused a gradual and significant increase ($P<0{\cdot}05$) in the signal for 72 kDa protein with a maximum at 8 h. LH addition to the incubation of diestrus tissues also showed a significant increase in the intensity of the signal of LHR protein up to 4 h with no further increase thereafter (Fig. 3C). β-Actin was used as a loading control.

Expression of 3β-hsd *mRNA in mouse endometrium*

To investigate whether nonpregnant mouse endometrium expresses *3β-hsd* mRNA, total RNA from endometrial and ovarian tissues (positive control) at metestrus were isolated

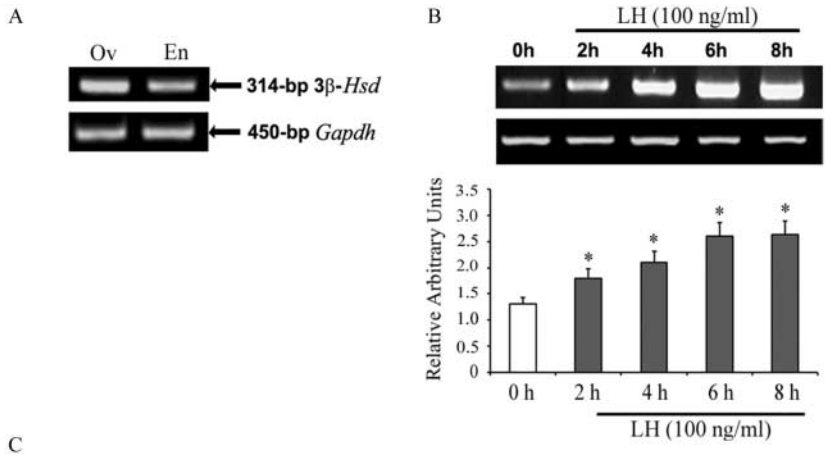


and RT-PCR was conducted with gene-specific primer of mouse 3β-HSD (Table 1). Figure 4A showed a prominent expression of *3β-hsd* mRNA both in ovary and in endometrium at this stage. *Gapdh* mRNA was used as loading control. To address the next obvious question about the LH induction of the expression of endometrial *3β-hsd* mRNA, tissues were incubated with LH (100 ng/ml) for different time intervals up to 8 h. Results shown in Fig. 4B demonstrate that LH administration resulted a significant ($P<0{\cdot}05$) increase in the expression levels of *3β-hsd* mRNA up to 6-h incubation with no further increase thereafter. The predicted size of the RT-PCR product of 3β-HSD was 314 bp. The nucleotide sequence of 314 bp fragments obtained from endometrium displayed a 99% homology with the comparable region of *M. musculus Hsd-3β1* (Budefeld *et al.* 2009; Fig. 4C).

Endometrial 3β-HSD enzyme and effects of LH

To determine whether 3β-HSD enzyme is present in mouse endometrium and LH has any stimulatory effect, tissues from all the four stages were incubated without (control) or with LH (100 ng/ml) in the presence of [^{3}H]P5 for 8 h and the rate of conversion of [^{3}H]P5 to [^{3}H]P_4 was measured. Figure 5A shows that the rate of formation of [^{3}H]P_4 in tissues from metestrus and diestrus was significantly higher ($P<0{\cdot}05$) compared with their respective control values after 8 h of incubation with LH. Tissues from metestrus stage mice, however, showed greater conversion ($P<0{\cdot}05$) than diestrus mice. Tissues from other stages showed poor response (Fig. 5A). As metestrus stage tissue showed the highest conversion, a similar experiment was done with the tissue of this stage in the presence of trilostane (a 3β-HSD inhibitor). Trilostane, at increasing concentrations (0–1·0 μg/ml), significantly inhibited ($P<0{\cdot}05$) LH-induced formation of [^{3}H]P_4 from [^{3}H]P5, almost in a dose-dependent manner (Fig. 5B). Trilostane alone had no inhibitory effect on basal level of conversion.

Figure 3 RT-PCR (A) and real-time PCR (B) amplification of mouse endometrium *Lhr* mRNA and effects of LH. RT-PCR was performed using 1 μg RNA from endometrium at various stages of the cycle (P, E, M, and D). Tissues were incubated for different time intervals up to 8 h with LH (100 ng/ml) with a control at 0 h without LH. *Gapdh* mRNA was used as loading control for RT-PCR and β-actin was used to normalize the abundance of *Lhr* mRNA in real-time PCR. Expression of LHR protein in mouse endometrium and effects of LH (C). Twenty micrograms of protein from soluble extract of endometrium at different stages of the cycle (P, E, M, and D) were separated on SDS–PAGE and electroblotted. β-Actin was used as a loading control. The pixel densities of the bands (lower panel, C) were quantified with ImageJ Software (National Institute of Health (NIH)) and have been represented in the bar diagram as relative arbitrary units considering the control value as 1. (A and C) are representative of four independent experiments, each performed in triplicate. Each bar in (B) represents the mean normalized expression (±S.E.M.) of three independent tissue pools (three mice per pool). *$P<0{\cdot}05$.



C

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       418
Hsd3b1 AATGGCCACGAGGAACAGAATCATGAAAGCACATGGTCTGATCCATACCCATACAGCAAA
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Uterus AATGGCCACGAGGAACAGAATCATGAAAGCACATGGTCTGATCCATACCCATACAGCAAA

Hsd3b1 AAGATGGCTGAGAAGGCAGTACTGGCAGCCAATGGGAGCATGCTGAAAAATGGTGGCACT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Hsd3b1 TTGAATACTTGTGCCTTAAGACCCATGTACATTTATGGGGAGAGAAGTCCATTCATTTTT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Hsd3b1 ATAGCCAACCCAGTATATGTGGAAAATGTGGCCTGGGCACACATTCTGGCAGCCAGGGGC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Uterus ATAGCCAACCCAGTATATGTGGAAAATGTGGCCTGGGCACACATTCTGGCAGCCAGGGGC
                  732
Hsd3b1 CTTCGAGACCCCAAG
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Uterus CTTCGAGACCCCAAG
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Figure 4 RT-PCR amplification of mouse endometrium 3β-HSD mRNA (A) and a time-course effect of LH there on (B). RT-PCR was performed using 1 μg RNA from ovarian (Ov) and endometrial (En) tissue at metestrus as described in Materials and Methods section. Endometrial tissues at metestrus were incubated for different time intervals up to 8 h with LH (100 ng/ml) with a control at 0 h without LH. GAPDH mRNA was used as loading control. The pixel densities of the bands (lower panel, B) were quantified with ImageJ Software (National Institute of Health (NIH)) and have been represented in the bar diagram as relative arbitrary units considering the control value as 1. (A and B) are representative of four independent experiments, each performed in triplicate. Values are mean ± S.E.M.; $*P<0{\cdot}05$. Automated nucleotide sequencing and homology analysis (C) between *Mus musculus* hydroxy-Δ-5-steroid dehydrogenase, 3β- and steroid Δ-isomerase 1 (*Hsd3b1*) mRNA (accession no. NM_008293.3) from nucleotide 418 to 731 and the PCR products were obtained using the upper primer described in the Materials and Methods section. A homology of 99% was found between the *M. musculus Hsd3b1* and the PCR product for a 314 bp fragment, suggesting that our amplified cDNA is complementary to *Hsd3b1* mRNA.

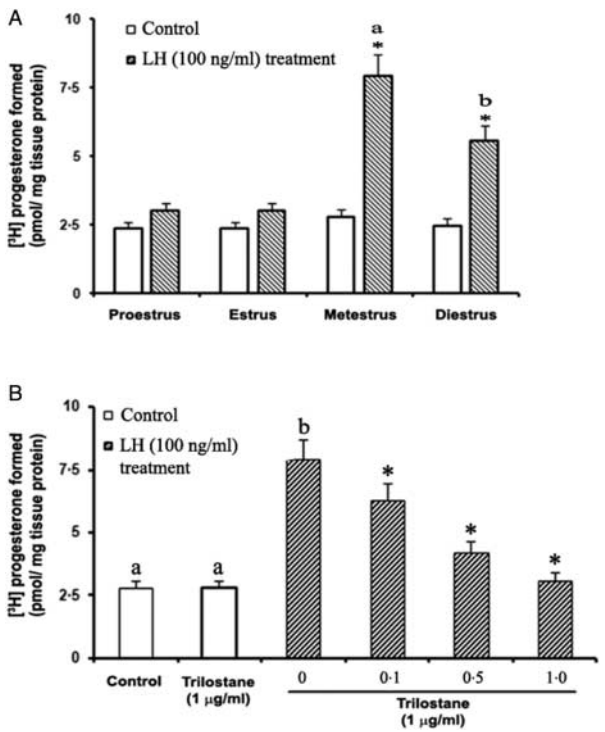

Figure 5 LH-induced conversion of [^{3}H]pregnenolone to [^{3}H]progesterone by mouse endometrium at four stages of estrous cycle (A). Tissues were incubated without (control) or with LH (100 ng/ml) for 8 h in the presence of [^{3}H]pregnenolone (2×10^6 c.p.m., 138 pmol). Effects of inhibitor of 3β-HSD, trilostane on LH-stimulated formation of [^{3}H]progesterone in endometrial tissue from metestrus stage mice (B). In all the experiments, values are the mean ± S.E.M. of four different experiments each performed in triplicate. *$P<0{\cdot}05$ compared with control. Different letters differ significantly ($P<0{\cdot}05$) from each other.

Dose–response and time-course effects of LH on P_4 production by mouse endometrium

To investigate whether mouse endometrium has the capacity to synthesize P_4 *de novo*, tissues from metestrus and diestrus stage were incubated for 12 h with increasing concentrations of LH (25–200 ng/ml) and media P_4 contents were measured. Figure 6A demonstrates that LH at increasing concentrations significantly ($P<0{\cdot}05$) augmented P_4 synthesis in tissues of both stages with a greater effect ($P<0{\cdot}05$) in metestrus. The optimal effective dose for LH was 100 ng/ml (Fig. 6A). Tissues from other stages showed poor response to LH (data not shown). A time-course study (2–12 h) was done in the absence (control) or presence of LH (100 ng/ml) with tissues from metestrus stage only. Figure 6B shows that LH-induced P_4 synthesis increased gradually and significantly ($P<0{\cdot}05$) with increasing time and maximum was recorded at 8 h incubation with no further increase thereafter.

Expression of Star *mRNA in mouse endometrium*

To further understand how LH enhanced endometrial steroidogenesis, RT-PCR analysis of *Star* mRNA levels was performed in endometrial tissues of metestrus mice. Incubation of endometrial tissue preparation with LH (100 ng/ml) for different time intervals up to 8 h resulted in a significant increase in *Star* mRNA expression at 4 h with a further increase at 6 h followed by a little decrease at 8 h (Fig. 7). The predicted size of the RT-PCR product of *Star* mRNA was 186 bp.

De novo *synthesized endometrial P_4 alone can maintain uterine growth*

Figure 8A shows the histological structure of intact saline (IC+S)-, OVX saline-, and OVX oil- controls as well as OVX+P_4-, and OVX+LH-treated mice uteri. It appears that a reduction in size of the uterus occurred due to ovariectomy in all the OVX-control groups compared with intact control, while both were greatly increased in response to either P_4 or LH treatment. This was more evident when uterine weight from all the individual experiments was

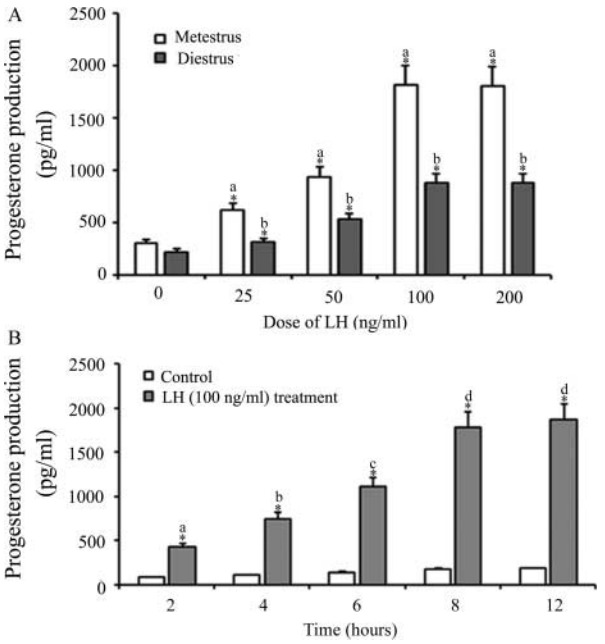

Figure 6 LH-stimulated progesterone production by mouse endometrium. Tissues from metestrus and diestrus stages were incubated with increasing concentrations of LH for 12 h and the media content of progesterone were estimated (A). Tissues were incubated without (control) or with LH (100 ng/ml) for different time intervals and the media content of progesterone were estimated (B). In all the experiments, values are the mean ± S.E.M. of four different experiments each performed in triplicate. *$P<0{\cdot}05$ compared with control. Different letters differ significantly ($P<0{\cdot}05$) from each other.

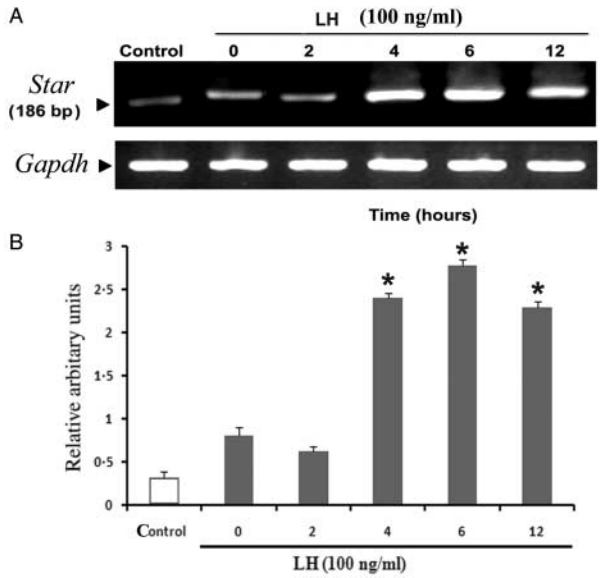

Figure 7 The effects of LH treatment on *Star* gene expression in mouse endometrial tissues. Endometrial tissue at metestrus was incubated for different time intervals up to 8 h with LH (100 ng/ml) with a control at 0 h without LH. GAPDH was used as the loading control. The pixel density (lower panel) of the bands was quantified with ImageJ Software. *$P<0·05$ compared with control.

recorded. Figure 8B shows a significant reduction ($P<0·05$) in uterine weight due to ovariectomy, which was significantly ($P<0·05$) increased after P_4 or LH treatment. To ascertain that increase in uterine weight was due to synthesis of protein, endometrial tissue from all the experiments were subjected to *in vitro* incubation separately for 6 h in the presence of ^{14}C leucine with other cold amino acids. Figure 8C shows a significant ($P<0·05$) reduction in trichloro acetic acid (TCA)-precipitable radioactivity due to ovariectomy both in saline- and oil-treated tissues, while this was significantly ($P<0·05$) increased after P_4 or LH treatment. Saline or oil alone had no inhibitory or stimulatory effects on TCA-precipitable radioactivity.

Second messenger activity

Effect of SQ22536 on LH-stimulated P_4 production Endometrial tissues from metestrus mice were incubated for 8 h in SF-DMEM with adenylate cyclase inhibitor SQ22536 (0·1–1·0 mM) and LH-stimulated P_4 production was examined. Table 2 shows that SQ22536 at increasing concentrations significantly ($P<0·05$) attenuated LH-stimulated P_4 production after 8 h incubation. The SQ22536 concentration at which maximum inhibition was recorded was 1·0 mM.

Effect of modulators of adenylate cyclase and PKA on P_4 production A similar experiment was conducted with increasing concentrations of FK (0·1–10·0 μM) or dbcAMP (0·1–10·0 mM) modulators of adenylate cyclase and PKA respectively, and P_4 contents in the media were examined. Table 2 demonstrates that FK and dbcAMP treatments gradually and significantly ($P<0·05$) increased endometrial P_4 production compared with their respective control values. The optimal effective doses of FK and dbcAMP were 10 μM and 10 mM respectively.

Effect of inhibitor of MEK1/2 on LH-induced P_4 production A role for MAPK activated signaling in LH-induced P_4 production was shown by incubating endometrial tissues from metestrus with LH and the MEK1/2 inhibitor, PD98059. For this, following 1·0-h pre-incubation with increasing doses of PD98059 (0·1, 1·0, and 5·0 mM), tissues were incubated with LH (100 ng/ml) for 16 h and steroid contents in the media were estimated. Figure 9A shows that PD98059 at increasing concentrations gradually and significantly ($P<0·05$) inhibited LH-induced P_4 production. At high concentration of PD98059 (5 μM), LH-induced P_4 production was not significantly different from its basal level. The concentration of PD98059 at which the maximum inhibition was noticed was 5 μM. PD98059 alone (5 μM) had no inhibitory effect on P_4 production.

Time-, concentration-, and MEK1/2-dependent activation of ERK1/2 by LH The activity of endometrial ERK1/2 was indirectly determined using a phospho-specific ERK1/2 antibody. Tissues from metestrus stage mice treated with increasing doses of LH (0, 25, 50, 100, or 200 ng/ml) for 120 min induced a dose-dependent increase in ERK1/2 phosphorylation (Fig. 9B). Tissues treated with LH (100 ng/ml) for 0, 5, 10, 20, 60, or 120 min showed

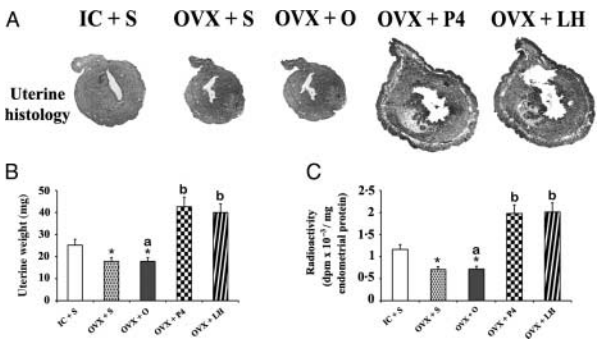

Figure 8 Histology (A), weight (B), and TCA-precipitable radiolabeled protein (C) of uterus dissected out from saline-injected intact control (IC+S) and ovariectomized (OVX+S); oil-injected OVX (OVX+O); P_4-injected OVX (OVX+P_4), and LH-injected OVX (LH+OVX) mice at metestrus stage (hematoxylin and eosin stain, 40×). Details of experiments have been described in the Materials and Methods section. For TCA-precipitable radiolabeled protein, uterine tissues from all the experiment groups were subjected to *in vitro* incubation in the presence of [^{14}C]leucine and other cold amino acids for 6 h (C). Each value is the mean±S.E.M. of four different experiments, each performed in triplicate. *$P<0·05$ compared to IC and OVX. Different letters differ significantly ($P<0·05$) from each other.

Table 2 Effect of modulators of adenylate cyclase (forskolin) and PKA (dbcAMP) on progesterone production and inhibitor of adenylate cyclase (SQ22536) on LH-stimulated progesterone production. Endometrial tissues from metestrus stage were incubated in DMEM containing various concentration modulators and/or inhibitors for 12 h. Tissues were pre-incubated for 1 h in the presence of inhibitor. Each value represents mean±S.E.M. of four experiments and comprises three replicate incubations of endometrial tissue obtained from single donor mice. 209×297 mm (300×300 dpi)

Control	Doses	Progesterone (pg/ml)
Control	–	307·5±24·11
Forskolin (μM)	0·1	656±41·06*
	1·0	1082·5±48·73*
	10	1810·5±103·2*
dbcAMP (mM)	0·1	550±32·78*
	1·0	865±70·6*
	10	1600±42·42*
LH (ng/ml)	100	1813·75±91·96*,a
LH (100 ng/ml)+	0·1	1470±53·78
SQ22536 (mM)	0·5	882·5±58·12b
	1·0	427·5±37·06c

*$P<0·05$ vs without LH, forskolin, or dbcAMP. Means with different letters differ significantly from each other.

increasing levels of phosphorylated ERK1/2 and strongest response to LH occurred between 60 and 120 min (Fig. 9C). The stimulatory effect of LH on phosphorylated ERK1/2 was not attributed to increased ERK protein levels, as total ERK1/2 protein was unaffected by incubation time and LH treatment (Fig. 9B, C and D; lower panel). Tissues treated with LH for 60 and 120 min in the presence of increasing concentrations of the MEK1/2 inhibitor, PD98059, blocked stimulatory effects of LH on ERK1/2 phosphorylation almost in a concentration-dependent manner (Fig. 9D).

Effects of SQ22536, FK, and dbcAMP on ERK1/2 phosphorylation and effects of PD98059 In a similar experiment, endometrial tissues from metestrus stage mice were treated for 1 h with PD98059 (5·0 μM) followed by incubation with LH (100 ng/ml), SQ22536 (1·0 mM), FK (10 μM), or dbcAMP (10 mM) for 120 min and ERK1/2 phosphorylation was examined. Figure 10 shows that treatment with LH, dbcAMP, or FK for 120 min increased the levels of ERK1/2 phosphorylation. The LH-induced increase in ERK1/2 phosphorylation was blocked by SQ22536. The MEK1/2 inhibitor PD98059 at concentration 5·0 μM reduced the basal level of active ERK1/2 and blocked LH-, dbcAMP-, and FK-induced increases in ERK1/2 phosphorylation (Fig. 10).

Discussion

The present work demonstrated that *Lhr* mRNA and its protein are present in nonpregnant mouse endometrium and both are expressed maximally at metestrus immediately after ovulation, when there is a high LH titer in mouse blood. Results also demonstrated that LH addition to the incubation can elevate their expression levels *in vitro*. The possible physiological significance of the presence of these LHRs was evident from the observation that LH increased the expression of both *3β-HSD* gene and 3β-HSD enzyme activity as well as *Star* gene expression, leading to increased *de novo* synthesis of P_4 in the mouse endometrium mainly at the metestrus stage. The function of P_4 in nonpregnant mice uterus is not yet known but may be related to its participation in the local regulation of P_4-dependent processes as shown in

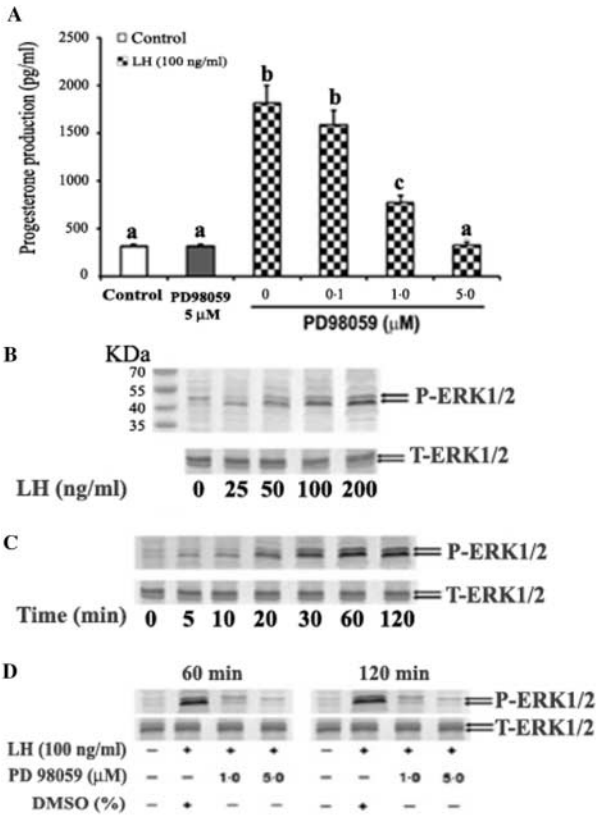

Figure 9 Effects of the inhibitor of MEK1/2, PD98059 on LH-stimulated progesterone production by mouse endometrium (A). Mean progesterone production by endometrial tissue at metestrus incubated for 12 h with plain medium (control), with 100 ng/ml LH and increasing concentrations of PD98059 or with PD98059 alone (A) is shown. Concentration-, time-, and MEK1/2-dependent activation of endometrial ERK1/2 by LH in endometrium at metestrus. Immunoblot analyses of phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (T-ERK1/2) in the tissues incubated with increasing concentrations of LH for 120 min (B), with 100 ng/ml LH for 5–120 min (C), or with 100 ng/ml LH, and increasing concentrations of PD98059 or with 0·1% DMSO vehicle for 60 and 120 min (D). Mobilities of molecular mass standard are given in kilodaltons on the left (B). Immunoblot analyses were performed at least three times with nearly identical results. Each value represents mean±S.E.M. of four different experiments, each performed in triplicate (A). Means with different letter differs significantly from each other ($P<0·05$).

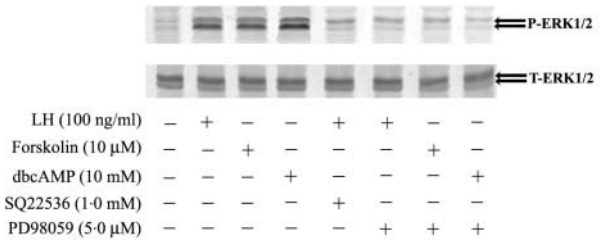

Figure 10 Effects of modulators of adenylate cyclase and PKA on ERK1/2 activation and inhibitor of adenylate cyclase (SQ22536) on attenuation of ERK1/2 phosphorylation in mouse endometrial tissue at metestrus stage. Immunoblot analyses of phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (T-ERK1/2) in the tissues incubated with LH, forskolin (FK), or dbcAMP or SQ22536 with or without PD98059 for 120 min are shown. Immunoblot analyses were performed at least three times with nearly identical results.

endometrium from OVX mice, where in the absence of ovarian steroid, *de novo*-synthesized P_4 can maintain normal endometrial growth.

Extragonadal LHRs have been described in the reproductive tract of several species. Since the first description of LHR/HCG receptor-like immunoreactivity in pig uterus (Ziecik *et al.* 1986), several groups have consequently confirmed these results with a variety of methods in different species (Bonnamy *et al.* 1989, Mukherjee *et al.* 1994, Derecka *et al.* 1995, Zheng *et al.* 2001, Licht *et al.* 2003). In uterine membrane preparation of rats and mice, highest binding levels were detected at metestrus and late diestrus stages (Bonnamy *et al.* 1989, Mukherjee *et al.* 1994). LHR in the uterus of pregnant rats (Bonnamy *et al.* 1993) and mice (Das *et al.* 2009) has also been demonstrated. In human uterus, although RT-PCR, northern, and western blot data have confirmed the presence of LHR/HCG receptor (Reshef *et al.* 1990, Han *et al.* 1997, Lin *et al.* 2005), their existence has been questioned by several groups. Stewart *et al.* (1999) proposed the expression of a variant form of uterine LHR, which would not only respond to classical ligands (LH and HCG) but also to TSH and FSH or even free glycoprotein α subunit (Stewart 2001). In mouse uterus, a binding study and *in situ* hybridization technique revealed that LHR is localized in endometrial stromal cells (Zheng *et al.* 2001). Several groups also proposed different paracrine functions of LH/HCG in endometrium-like prostaglandin and cytokine biosynthesis (Zhoi *et al.* 1997, Rao 1998, Zhou *et al.* 1999, Srisuparp *et al.* 2003), and modulation of decidualization and implantation of the embryo into the uterus (Han *et al.* 1999, Banaszak *et al.* 2000, Licht *et al.* 2002, Das *et al.* 2009). The present study using RT-PCR and real-time PCR demonstrates a clear and distinct expression of *Lhr* mRNA in mouse endometrium at each stage of the estrous cycle with a maximum at metestrus. Our data are the first to detect and quantitate *Lhr* mRNA using both these techniques in mouse endometrium at all stages of the reproductive cycle.

The nucleotide sequence homology between our PCR-amplified products (202 bp) was 99% identical to the comparable region of the *M. musculus Lhcgr* mRNA, showing that our amplified cDNA was complementary to the *M. musculus Lhr* mRNA at nucleotides 1675–1877. To further confirm the presence of LHR in mouse endometrium, we have sequenced the full-length PCR product obtained by RACE. It is interesting to observe that the endometrial *Lhr* mRNA sequence is 100% identical to the mouse ovarian *Lhr* mRNA sequence. This suggests that both the tissues may have the same gene and they produce the same product.

The specific antibody to mouse LHR protein detected a 72 kDa LHR band of similar size both in endometrial and in ovarian tissues, suggesting the presence of the same form of receptor protein on their cell surface in both the tissues. A strong signal for the 72 kDa LHR was present in endometrium from metestrus; however, there was only weak signal in other stages. All these demonstrate a good correlation between *Lhr* mRNA expression and induction of LHR in mouse endometrium at metestrus.

In the RT and real-time PCR data, we observed gradual and significant stimulatory effects of LH on endometrial *Lhr* mRNA expression at metestrus from 4 to 8 h incubation. The time-course study also indicated similar stimulatory effects of LH on the expression of LHR protein at metestrus. Information on gonadotropin-dependent expression of *Lhr* mRNA and its protein in the uterine tissue of any animals has not yet been available. Thus, our data may be considered as the first to detect LH-dependent expression of *Lhr* mRNA and its protein in mouse endometrium.

The functional role of the presence of LHR in mammalian uterus is not fully known, but investigators have speculated that LHR may be functionally important in decidualization and implantation of embryo in the uterus (Han *et al.* 1997, 1999). Lei *et al.* (1992) suggested that LHR may be involved in vascular dilation resulting in blood flow change in human uterus during the reproductive cycle and pregnancy. Rao (1998) demonstrated that LH is able to induce cyclooxygenase 2 gene expression both in glandular epithelium and in stromal cells in human endometrium resulting in increased prostaglandin production (Zhoi *et al.* 1997). The reported rise of uterine LHR/HCG receptor at metestrus in rats after ovulation (Bonnamy *et al.* 1990) may be due to modulation of P_4 content (Bonnamy *et al.* 1989). Our previous study also showed LH-induced increases in P_4 synthesis in mouse uterus at metestrus (Debnath *et al.* 2000). Thus, the maximum expression of LHR in mouse endometrium at metestrus in this study corroborates the earlier findings with rat uterus, indicating that this rise was due to post-ovulatory production of P_4 in mouse endometrium.

We detected *3β-hsd* mRNA expression in nonpregnant mouse endometrium at metestrus. Nucleotide sequence homology between our RT-PCR product consisting of 314 bp was 99% identical to the comparable region of mouse *3β-hsd* mRNA, showing that our amplified cDNA was complementary to the *M. musculus* mRNA *3β-hsd1* at nucleotides 418–731 bp. The 314 bp cDNA was also present in mouse ovarian tissue at metestrus, suggesting that both

ovary and endometrium have the same *3β-hsd* gene. The results thus demonstrate the presence of functional *3β-hsd* mRNA in mouse endometrium at this stage. *3β-hsd* mRNA expression in mouse endometrium during early pregnancy has already been documented (Peng *et al.* 2002, Das *et al.* 2009) and demonstration of the expression of this mRNA in nonpregnant mouse endometrium at metestrus in this study may be the first one in any mammalian species. The LH-induced increase in the activity of 3β-HSD enzyme in endometrium at metestrus after 8-h incubation demonstrates translation of *3β-hsd* mRNA. Activity of 3β-HSD enzyme has been described in the endometrium of several species, either by conversion study during follicular (Sweat & Bryson 1969) and luteal phase (Henricks & Tindall 1971) or histochemically during maternal recognition of pregnancy (Flood & Ghazi 1981). Our previous study also reported LH-induced stimulation of 3β-HSD enzyme activity in mouse uterus (Debnath *et al.* 2000). In this study, there is a clear indication for the stimulation of *3β-hsd* mRNA expression and its enzyme activity leading to increased *de novo* P_4 synthesis by nonpregnant mouse endometrium after LH stimulation particularly at metestrus. LH- and FSH-induced steroidogenesis in ovarian theca and granulosa cells is mediated by common signaling molecules including cAMP and steroidogenic factor-1 (SF-1). SF-1 regulates the expression of genes that encode *Star*, *P450scc*, *3β-hsd*, and *P450arom* (Bulun & Adashi 2002). Thus, SF-1 can be regarded as a downstream master switch that orchestrates ovarian steroidogenesis. In this study, SF-1 activity has not been measured but RT-PCR demonstrated the presence and enhanced expression of the *Star* gene after LH stimulation in mouse endometrium that might link cAMP and stimulation of 3β-HSD activity in such tissue. All these findings indicate that mouse endometrium at metestrus is able to synthesize P_4 *de novo*. As *Lhr* mRNA and protein are expressed maximally at metestrus and LH can stimulate their expression, the LH-induced augmentation in the production of P_4 at this stage demonstrates the presence of functional LHR mRNA in mouse endometrium.

The physiological significance of this locally produced hormone remained unclear in the context of normal circulatory levels of P_4 in cycling mouse. In rats, there are two P_4 maxima – a brief preovulatory peak, believed to be secreted by large follicles, and a lesser but more prolonged one at the time of corpora luteum formation (Gorbman *et al.* 1982). We observed increased *de novo* P_4 synthesis at metestrus when corpus luteum formation takes place. It has been documented that uterine growth and differentiation after ovulation is regulated by P_4/estrogen interaction. P_4 appears to have multiple functions, the most important being endometrium preparation for implantation and maintenance of pregnancy (Pepe & Albrecht 1995). The corpus luteum is the primary source of P_4. We observed that ovariectomy of mice at metestrus reduced uterine weight, which increased sufficiently after LH or P_4 treatment. High endogenous gonadotropin release from the pituitary after ovariectomy might have a role in uterine growth, but diminished growth and protein synthesis of the uterus after ovariectomy in mice indicates that the rise in endogenous LH may not be sufficient to maintain the growth of the uterus. Administration of exogenous LH is therefore required for normal growth. For a clear understanding of the role of endogenous LH, it is essential to measure the quantity of circulatory LH after ovariectomy. Interestingly, LH can stimulate endometrial protein synthesis in OVX mice to about threefold over the OVX control. These results suggest that LH stimulates endometrial protein synthesis either directly or via the mediation of P_4 hormone. P_4 treatment to OVX mouse also increased endometrial protein synthesis, indicating that *de novo* synthesized P_4 might have a role in increased protein synthesis. A time-course study of the effect of LH on the increase in protein synthesis and P_4 production by the endometrial tissue may elucidate whether maintenance of endometrial growth in OVX mice is solely caused by stimulating P_4 production in endometrium. The uteri of transgenic mice lacking *Lh* gene expression are only 30% the weight of the WT control mice and have an infantile appearance (Lee *et al.* 1966), suggesting that LH has a stimulatory role on endometrial growth maybe via P_4 production.

Consistent with earlier observations in mammalian gonads (see review, Leung & Steele (1992)), increased P_4 synthesis by mouse endometrium at metestrus in the presence of FK and dbcAMP, modulators of adenylate cyclase and PKA respectively, and inhibition of LH-stimulated P_4 synthesis in the presence of the adenylate cyclase inhibitor, SQ22536, demonstrate a possible involvement of adenylate cyclase and PKA in mouse endometrial steroidogenesis. In this study, cAMP levels after LH treatment were not measured, but increased basal and LH-, as well as FK- and dbcAMP-induced steroid production, may suggest that LH activates the receptor, which is known to act through a cAMP-dependent pathway. A similar signaling pathway has also been reported in HCG-induced P_4 synthesis in rat uterus (Bonnamy *et al.* 1989). To conclude the involvement of cAMP–PKA pathway in LH signaling in mouse endometrial steroid production, future studies using specific inhibitors of PKA and measurement of cAMP after incubation of endometrial tissues with LH are essential.

The results of this study clearly show a second signaling pathway involving MEK1/2 and ERK1/2 in LH-induced endometrial P_4 production. We observed that LH treatment increased ERK1/2 phosphorylation in a dose- and time-dependent manner. A role for MAPK/ERK signaling in regulating gonadotropin-induced steroidogenesis in mammalian and avian granulosa cells (Moore *et al.* 2001, Seger *et al.* 2001, Dewi *et al.* 2002, Cottom *et al.* 2003, Su *et al.* 2006, Woods & Johnson 2007) and in rat Leydig cells (Martinelle *et al.* 2004) has also been reported. Although LH-induced P_4 synthesis by the mouse/rat uteri has been documented earlier (Bonnamy *et al.* 1989, Mukherjee *et al.* 1994, Debnath *et al.* 2000), little is known about its mechanism of action. To our knowledge, demonstration of MAPK signaling in

LH-induced P_4 synthesis in mouse endometrium may be the first one in any non-gonadal tissues of mammals. Thus, signaling for LH-induced endometrial steroidogenesis in mice shares a common pathway in mediating LH-induced ovarian steroid production in mammals and aves. We further observed that LH-stimulated ERK1/2 phosphorylation leading to increased P_4 production is significantly inhibited by PD98059, suggesting that action of LH on ERK1/2 phosphorylation is mediated by upstream MEK1/2. Although involvement of MAPK in mediating gonadotropin-stimulated steroidogenesis has been observed in many species, conflicting results in different steroidogenic tissues have been demonstrated (Seger *et al.* 2001, Dewi *et al.* 2002, Manna *et al.* 2002, 2006, Seto-Young *et al.* 2003, Martinelle *et al.* 2004, Tajima *et al.* 2005). For example, inhibition of MAPK/ERK1/2 activity with PD98059 and U0126 has been shown to be associated with stimulation (Seger *et al.* 2001, Tajima *et al.* 2003), inhibition (Gyles *et al.* 2001, Manna *et al.* 2002, Martinelle *et al.* 2004), or no effect (Tai *et al.* 2001, Seto-Young *et al.* 2003, Tajima *et al.* 2005) on steroidogenic response. Taken together, these findings demonstrate a complex role for the MAPK/ERK cascade in the regulation of the steroidogenic response that appeared to be tissue and stimulus specific. The mechanism by which gonadotropin binding to its GPCRs triggers activation of the MEK/ERK pathway is still controversial. As suggested previously (Pierce *et al.* 2001, Kim *et al.* 2002, Drube *et al.* 2006, Evaul & Hammes 2008), it may be possible that gonadotropin binding to GPCRs in mouse endometrium activates the MEK/ERK pathway through transactivation of EGFRs and further studies would need to explore the actual mechanism of such transactivation.

Involvement of cAMP in LH-stimulated activation of ERK in mouse endometrium is demonstrated by showing inhibition of ERK1/2 phosphorylation in the presence of the adenylate cyclase inhibitor, SQ22536. Similar cAMP-dependent ERK1/2 phosphorylation has been demonstrated in mammalian granulosa cells (Seger *et al.* 2001, Dewi *et al.* 2002, Cottom *et al.* 2003). Attenuation of FK- and dbcAMP-stimulated ERK1/2 phosphorylation and steroid production by PD98059 indicate that the function of the MEK/ERK pathway is likely to be distal to adenylate cyclase and PKA. As we have not used any PKA inhibitor, involvement of PKA in LH-stimulated ERK1/2 phosphorylation is not clear in this study. One mechanism that activates the ERK cascade independent of PKA includes activation of cAMP-responsive guanosine nucleotide exchange factor for small GTPase such as Rap1 and Rap2. On binding with cAMP, these components rapidly activate Rap1 and promote the activation of B-Raf and the rest of the ERK cascade (de Rooij *et al.* 1998, Richards 2001). It is therefore possible that the major signaling pathway regulating endometrial steroidogenesis in this study including adenylate cyclase and MAPK-dependent pathways converge at a point distal to activation of PKA and ERK1/2. The possible target for coordinated regulation of this signaling pathway may be the STAR protein and/or transcription factors regulating its synthesis because there is evidence that in steroidogenic cells, cAMP, PKA, and MAPK can modulate the activity of this protein or associated transcription factors (Cherradi *et al.* 1997, Manna *et al.* 1999, Tajima *et al.* 2003, Evaul & Hammes 2008). In our present study, we also observed a stimulatory role of *Lh* on *Star* gene expression in mouse endometrium for P_4 production. Available information also indicated that in gonads and adrenal tissue, there is a phosphorylation of STAR by the PKA–MEK1/2–ERK1/2 pathway that is necessary for normal steroid synthesis (Gyles *et al.* 2001, Poderoso *et al.* 2008).

In this study, it was found that levels of mouse endometrial LHR mRNA and protein varied during the estrous cycle with the maximum at metestrus. Exogenous LH has a stimulatory effect on the expression of both receptor mRNA and protein at metestrus. LH could elevate P_4 synthesis in the endometrium at metestrus, and this *de novo* synthesized steroid might have a role in growth and differentiation of endometrium immediately after ovulation. This study also shows that multiple but independent signaling pathways are operative in LH-induced endometrial steroidogenesis. The role of the MAPK signaling cascade involving MEK1/2 and ERK1/2 in the regulation of LH-induced P_4 production by mouse endometrium has been identified.

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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