EPAC2-mediated calreticulin regulates LIF and COX2 expression in human endometrial glandular cells

Kazuya Kusama1,2*, Mikihiro Yoshie1,*, Kazuhiro Tamura1, Kazuhiko Imakawa2 and Eiichi Tachikawa1

1Department of Endocrine and Neural Pharmacology, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
2Laboratory of Theriogenology and Animal Breeding, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
*(K Kusama and M Yoshie contributed equally to this work)

Correspondence should be addressed to K Tamura
Email hiro@toyaku.ac.jp

Abstract

The proper production of the implantation-related factors, leukemia inhibitory factor (LIF), cyclooxygenase 2 (COX2, PTGS2), and prostaglandin E2 (PGE2) in the uterine glands is essential for embryo implantation and the establishment of endometrial receptivity. It has been shown that cAMP-mediated protein kinase A (PKA) signaling regulates the production of these factors. We have previously reported that exchange protein directly activated by cAMP 2 (EPAC2, RAPGEF4), another cAMP mediator, is involved in the differentiation of endometrial stromal cells through the regulation of the expression of calreticulin (CALR). To address whether EPAC2–CALR signaling is involved in the expression of implantation-related factors, we examined the effect of EPAC2 and CALR knockdown on their expression in cultured human endometrial glandular epithelial EM1 cells, treated with forskolin, an adenylyl cyclase activator, an EPAC-selective cAMP analog (8-(4-chlorophenylthio)-O-methyl cAMP (CPT)), or a PKA-selective cAMP analog (N6-phenyl-cAMP (Phe)). In addition, the status of cell senescence was examined. EPAC2 knockdown suppressed the expression of CALR protein and mRNA in EM1 cells. Forskolin- or Phe-, but not CPT-, induced expression of LIF or PTGS2 and secretion of PGE2 was inhibited in EPAC2-or CALR-silenced EM1 cells. In addition, knockdown of EPAC2 or CALR increased senescence-associated beta galactosidase activity and expression of p21 but decreased expression of p53. These findings indicate that expression of CALR regulated by EPAC2 in endometrial glandular epithelial cells is critical for the expression of LIF and PTGS2-mediated production of PGE2 through cAMP signaling. Furthermore, EPAC2 and CALR could play a role in the maintenance of gland function.

Key Words

- exchange protein directly activated by cAMP (EPAC)
- calreticulin (CALR)
- leukemia inhibitory factor (LIF)
- cyclooxygenase 2 (COX2, PTGS2)
- prostaglandin E2 (PGE2)
- endometrial glandular epithelial cell

Introduction

Sex steroid hormones regulate the structure and function of the human uterine endometrium throughout the menstrual cycle (Lim & Wang 2010). This uterine change, including structural and secretory transformations, is a prerequisite for the establishment of uterine receptivity, peri-implantation embryo survival, and embryo implantation as well as placentation (Cha et al. 2012). Endometrial glandular epithelial cells synthesize and secrete implantation-related factors, including leukemia inhibitory factor (LIF), cyclooxygenase 2 (COX2, PTGS2),...
and prostaglandin E2 (PGE2), which are essential for embryo development and endometrial stromal cell differentiation (Stewart et al. 1992, Lim et al. 1999). LIF expression and PTGS2-mediated PGE2 production are elevated in endometrial glandular cells during the implantation window (Senturk & Arici 1998, Marions & Daniëls 1999, Milne et al. 2001). It has been demonstrated that Lif-null mice are infertile because of defective attachment of blastocysts to the uterine luminal epithelium (Chen et al. 2000), while Ptgs2-null mice show a failure in the decidualization of stromal cells (Matsumoto et al. 2002). In addition, activation of cAMP-mediated protein kinase A (PKA) signaling increases the expression of LIF and PTGS2 in human endometrial glandular cells (Zhou et al. 1999, Kutsukake et al. 2010). The proper expression of these factors is necessary for the successful establishment of pregnancy.

Exchange protein directly activated by cAMP (EPAC) is a cAMP-mediated signaling effector whose function is distinct from the classical PKA-signaling pathway (Kawasaki et al. 1998, de Rooij et al. 1998). EPAC has two isoforms, EPAC1 (RAPGEF3) and EPAC2 (RAPGEF4), which are implicated in several cellular responses, including cell proliferation, cell differentiation, hormone secretion/exocytosis, and formation of cell–cell junctions (Ahn et al. 2006, Bos 2006, Kooistra et al. 2007). In addition, EPAC1 is associated with the cAMP-dependent differentiation of cytotrophoblasts into syncytiotrophoblasts during placentation (Yoshie et al. 2010). We recently demonstrated that both EPAC1 and EPAC2 are expressed at the endometrial stroma, and glandular and luminal epithelium throughout the menstrual cycle, and activation of EPAC signaling enhanced decidualization of human endometrial glandular cells (Yoshie et al. 2010). The proper expression of these factors is necessary for the successful establishment of pregnancy.

To study a possible role of EPAC2 or CALR in the production of implantation-related factors, an immortalized human endometrial glandular epithelial cell line (EM-E6/E7/TERT-1 cells; EM1) was used. Cells were grown at 37 °C in DMEM and Ham’s F-12 supplemented with 10% (w/v) charcoal-stripped fetal bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 0.5 μg/ml amphotericin B. EM1 cells were treated with 15 μM forskolin, 200 μM CPT, and/or 200 μM Phe for 48 h.

**Materials and methods**

**Reagents and antibodies**

8-(4-Chlorophenylthio)-2’-O-methyl cAMP (CPT) and N6-phenyl-cAMP (Phe) were purchased from the Biolog Life Science Institute (Bremen, Germany). An adenyllyl cyclase stimulator, forskolin, and a selective PTGS2 inhibitor, NS-398, were obtained from Applichem (Darmstadt, Germany) and Cayman Chemical Company (Ann Arbor, MI, USA) respectively. Antibodies against CALR, p53 (clone 1C12), p21 (clone DCS60), and EPAC2 (clone 5B1) were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, clone GAPDH-71.1) was purchased from Sigma–Aldrich.

**Cell culture**

To study a possible role of EPAC2 or CALR in the expression of implantation-related factors, an immortalized human endometrial glandular epithelial cell line (EM-E6/E7/TERT-1 cells; EM1) was used. Cells were grown at 37 °C in DMEM and Ham’s F-12 supplemented with 10% (w/v) charcoal-stripped fetal bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 0.5 μg/ml amphotericin B. EM1 cells were treated with 15 μM forskolin, 200 μM CPT, and/or 200 μM Phe for 48 h.

**Treatment of EM1 cells with siRNA or PTGS2 inhibitor**

EM1 cells grown to 50% confluency in 24-well plates were transfected with non-targeting control siRNA (30 pmol/well, Qiagen), EPAC1 siRNA (30 pmol/well; S’-AUUGAGAUAUCUUCUGCUCCUUGAGG-3’ and S’-CUCCAUAGGAGCAGAAUCAUAU-3’, Invitrogen), EPAC2 siRNA (30 pmol/well; S’-UGUUCUUUAAGUCAGUCUGAUAUUG-3’ and S’-GGAACAGACUCAUAAAAGAACA-3’, Invitrogen), RAP1 siRNA (30 pmol/well; sc-36384D, Santa Cruz Biotechnology), or CALR siRNA (30 pmol/well; S’-GCAGACAAGCAGCAUGACGCUCUUU-3’ and S’-AAAAGCUUGAUAUGCUUGUCUCUC-3’, Invitrogen) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions. After treatment with siRNA for 24 h, the EM1 cells were washed with PBS and then cultured in fresh medium for 24 h, followed by medium containing or not containing forskolin or cAMP analogs for 48 h. The expression of LIF and PTGS2 mRNA peaked at between 48 and 72 h in the preliminary experiment.
The concentrations of siRNA sufficient to suppress the expression of each protein were predetermined as described previously (Kusama et al. 2014a) and the knockdown efficiency was 79.0±8.1% for EPAC2 and 75.0±8.5% for CALR at the protein level. In addition, the amounts of PTGS2 sufficient to inhibit the effect of PTGS2 were also determined. A dose of 10 µM NS-398 was used to treat EM1 cells, which were cultured for 49 h before RNA extraction.

**Immunoblotting**

Protein lysates from EM1 cells were prepared with RIPA buffer (Cell Signaling Technology) according to the manufacturer’s instructions. Equal amounts of protein (20 µg) were separated by SDS–PAGE and electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA, USA). These membranes were probed with primary antibodies against CALR (1:2000), p53 (1:1000), p21 (1:1000), or EPAC2 (1:2000). Immunoreactive bands were detected using ECL (PerkinElmer Life Science, Inc., Boston, MA, USA) after incubation with HRP-labeled goat anti-mouse or anti-rabbit IgG (0.5 g/ml, Vector Laboratories, Burlingame, CA, USA). Membranes were then treated with stripping solution (25 mM glycine–HCl, pH 2.0, containing 1% (w/v) SDS) and re-probed with an antibody against GAPDH (0.2 µg/ml). The relative band intensity was assessed by densitometric analysis of digitalized autographic images using Scion Image Software (Scion Corp., Fredrick, MD, USA) and normalized to GAPDH (Kusama et al. 2014a).

**RNA extraction and real-time RT-PCR**

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. RNA (100 ng) was amplified by real-time RT-PCR using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories). Sense (S) and antisense (AS) primers were 5'-GACCTCTGGCAGGTCAAGTC-3' (S) and 5'-TCACGTATGCTCATCGT-3' (AS) for CALR, 5'-TGAATGTCAGCCCATATGAA-3' (S) and 5'-TTCCAGTGCGAAGCA-3' (AS) for PTGS2, and 5'-AGCCA-CATCGCTCAGACA-3' (S) and 5'-GCCCAATTGAC-CAAATCC-3' (AS) for GAPDH. The fold change in the expression of each gene was calculated using the ΔΔCt method with GAPDH as an internal control (Kusama et al. 2014a).

**PGE2 ELISA**

EM1 cells were treated with control, EPAC2, or CALR siRNA for 24 h and then with forskolin, Phe, and/or CPT for 48 h. The culture medium was centrifuged at 10 000 g for 10 min at 4 °C, and the concentration of PGE2 in the supernatant was determined using a sandwich ELISA Kit (Prostaglandin E2 Express EIA Kit, Cayman Chemical Company) according to the manufacturer’s instructions.

**Senescence-associated beta galactosidase staining**

EM1 cells, transfected with control, EPAC2, or CALR siRNA for 48 h, were fixed in 4% (w/v) paraformaldehyde for 10 min and then washed twice with PBS. Staining was performed overnight at 37 °C in PBS (pH 6.0) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mM MgCl2, and 1 mg/ml X-gal. The number of senescence-associated beta galactosidase (SA-β-Gal)-positive cells was determined in five randomly chosen

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**Figure 1**

Downregulation of CALR expression in EPAC2-silenced EM1 cells. EM1 cells were treated for 24 h with non-targeting control (Cont) or EPAC2 siRNA and cultured in fresh medium for an additional 24 h. (A) Cell lysates were subjected to immunoblot analysis with an anti-CALR or EPAC2 antibody. The same blot was stripped and re-probed with an anti-GAPDH antibody, which served as a loading control. (B) Total RNA was subjected to real-time RT-PCR analysis to determine the relative levels of CALR normalized to levels of GAPDH from three independent experiments. *P < 0.01 versus Cont siRNA.
fields and expressed as a ratio compared with the control siRNA-transfected group (Kusama et al. 2014a).

Statistical analysis

Data are expressed as the mean±s.e.m. Significance was assessed using the Tukey–Kramer multiple comparisons test. A P value <0.05 was considered statistically significant.

Results

Downregulation of CALR expression in EPAC2-silenced EM1 cells

We previously reported that EPAC2 modulated expression of CALR in human endometrial stromal cells (Kusama et al. 2014a). To determine whether knockdown of EPAC2 downregulated expression of CALR protein, the level of CALR in EPAC2-silenced EM1 cells was examined by immunoblot analysis. EPAC2 knockdown significantly suppressed CALR expression (Fig. 1A). In addition, results from real-time RT-PCR analysis indicated that knockdown of EPAC2 inhibited the expression of CALR mRNA (Fig. 1B).

Inhibition of expression of implantation-related factors in EPAC2- or CALR-silenced EM1 cells

It is reported that cAMP-mediated PKA activation increases the expression of LIF or PTGS2 mRNA (Zhou et al. 1999, Kutsukake et al. 2010). We investigated whether EPAC2 and CALR were involved in cAMP signaling-induced expression of LIF or PTGS2 and secretion of PGE2 in EM1 cells. Transfection of EM1 cells with CALR or EPAC2 siRNA suppressed expression of CALR or EPAC2 protein respectively (Fig. 2A). The effect of forskolin, an adenylyl cyclase activator, on expression of LIF or PTGS2 was reduced in CALR- or EPAC2-silenced EM1 cells (Fig. 2B and C). Forskolin-induced secretion of PGE2 was also blocked by knockdown of CALR or EPAC2 (Fig. 2D). To confirm that these results were EPAC2- or CALR-specific, EM1 cells transfected with EPAC1 or RAP1 siRNA were similarly treated with forskolin. Forskolin-induced expression of LIF or PTGS2 did not differ in EPAC1- or RAP1-silenced EM1 cells (Fig. 2E and F). These results indicated that EPAC2 and CALR, but not EPAC1 or RAP1, regulated the cAMP-stimulated expression of LIF and PTGS2 and secretion of PGE2 in EM1 cells. PGE2 has

Figure 2

Inhibition of the expression of forskolin-stimulated implantation-related factors in EPAC2- or CALR-silenced EM1 cells. (A, B, C, D, E and F) EM1 cells were treated for 24 h with non-targeting control (Cont), EPAC2, CALR, EPAC1, or RAP1 siRNA and cultured for an additional 24 h. EM1 cells were then treated with forskolin (15 μM) for 48 h. The expression of EPAC2 or CALR was determined by immunoblotting (A). The same blot was stripped and re-probed with an anti-GAPDH antibody, which served as a loading control. Total RNA was subjected to real-time RT-PCR analysis to determine the levels of LIF (B and E) and PTGS2 (C and F) mRNA. GAPDH was used as an internal control. (D) The level of PGE2 in culture media was determined by ELISA. (G) One hour before the forskolin stimulation, EM1 cells were treated with or without 10 μM NS-398. EM1 cells were further cultured for 48 h and harvested for extraction of RNA, from which levels of LIF and PTGS2 mRNA were analyzed. (B, C and D) Cont siRNA (white bars), Epac2 (black bars), and CALR siRNA (gray bars), (E and F) Cont siRNA (white bars), EPAC1 (gray bars), and RAP1 siRNA (black bars). (G) Control (white bars) and NS-398 (gray bars). The results from three independent experiments are presented. *P<0.01 versus Cont siRNA and #P<0.01 versus Cont. Values represent the mean±s.e.m.
been shown to activate the intracellular downstream pathways through cAMP in endometrial stromal cells (Cheng et al. 2007). We therefore examined whether treatment with NS-398, a PTGS2 inhibitor, affected the levels of LIF and PTGS2 mRNA in EM1 cells treated with forskolin. However, the expression of LIF and PTGS2 induced by forskolin was not affected by the presence of a PTGS2 inhibitor (Fig. 2G).

**Induction of cAMP analog-stimulated implantation-related factors was inhibited in EPAC2- or CALR-silenced EM1 cells**

Results of previous studies indicated that EPAC2 signaling potentiated the differentiation of endometrial stromal cells when these cells were stimulated by a PKA-selective agonist (Kusama et al. 2013, 2014a). To investigate the role of EPAC2- or CALR-mediated cAMP signaling in the cAMP-mediated induction of implantation-related factors, EM1 cells in which EPAC2 or CALR was downregulated were treated with PKA-selective (Phe) and/or EPAC-selective (CPT) cAMP analogs. The expression of LIF or PTGS2 was increased by Phe, but not by CPT (Fig. 3A and B). Moreover, LIF or PTGS2 expression when cells were treated with Phe and CPT (Phe/CPT) was similar to that of the Phe treatment group. Thus, the EPAC agonist did not upregulate the expression of LIF and PTGS2 when these cells were stimulated with a PKA agonist. In CALR- or EPAC2-silenced EM1 cells, Phe- or Phe/CPT-induced expression of LIF or PTGS2 was significantly downregulated. Similar results were observed for the secretion of PGE2 (Fig. 3C). These results indicated that, regardless of EPAC signaling, EPAC2 and/or CALR regulate the expression of LIF and production of PGE2 through the expression of PTGS2 in EM1 cells.

**Induction of senescence in EPAC2- or CALR-silenced EM1 cells**

It has been reported that cell senescence is induced in Calr-deficient mouse fibroblasts (Iakova et al. 2004), and that knockdown of EPAC2 or CALR promotes cell senescence in endometrial stromal cells (Kusama et al. 2014a). To investigate whether EM1 cells in which EPAC2 or CALR had been downregulated exhibited cell senescence, we examined the activity of SA-β-Gal, and the expression of p21 and p53, well-established markers of cellular senescence (Kusama et al. 2014a). The intensity of SA-β-Gal staining significantly increased in the EPAC2 or CALR siRNA-treated EM1 cells compared with the control cells (Fig. 4A and B). Furthermore, knockdown of either EPAC2 or CALR in EM1 cells suppressed the level of p53 but increased the level of p21 (Fig. 4C, D and E). These results were indicative of an association between EPAC2/CALR and senescence in EM1 cells.
Discussion

To elucidate the possible role of EPAC2 or CALR in the endometrial gland as it prepares for implantation, we investigated the expression of LIF and PTGS2 and the secretion of PGE2 in EPAC2- or CALR-silenced endometrial epithelial EM1 cells. EM1 cells exhibit gland-like structure in Matrigel and respond to 17β-estradiol by proliferation, both of which are characteristic of epithelial cells, not stromal cells (Kyo et al. 2003, Kutsukake et al. 2010). The results of this study indicated that knockdown of EPAC2 suppressed the expression of CALR mRNA and protein and that EPAC2-regulated CALR was essential for the production of LIF and PTGS2 and secretion of PGE2; however, forskolin-induced of these implantation-related factors was inhibited in EPAC2- or CALR-silenced EM1 cells. These results indicated that although the activation of EPAC signaling itself had no effect, EPAC2 or CALR was required for sufficient expression of LIF and PTGS2 and production of PGE2 in endometrial glandular epithelial cells. It has been shown that PGE2 itself activates the intracellular downstream pathways through cAMP in endometrial stromal cells (Cheng et al. 2007). However, expression of LIF induced by forskolin was not altered in the presence of a PTGS2 inhibitor in EM1 cells, indicating that expression of LIF mediated through activation of PKA could occur independently from endogenous PGE2. It has also been reported that knockdown of EPAC2 or CALR inhibits PKA signaling in human and rat endometrial stromal cells (Kusama et al. 2013, 2014a,b). In this study, the expression of PKA-selective cAMP analog (Phe)-stimulated implantation-related factors was inhibited in EPAC2- or CALR-silenced EM1 cells. Thus, these results indicate that EPAC2 or CALR could have interacted with the PKA-signaling pathway; however, the precise mechanisms by which EPAC2 or CALR affected the PKA-mediated effects on the expression of implantation-related factors is yet to be determined.

As shown in Fig. 4C and E, knockdown of EPAC2 or CALR suppressed the expression of p53 in EM1 cells. It has

![Figure 4](https://example.com/figure4.png)

**Figure 4**

Induction of senescence in EPAC2- or CALR-silenced EM1 cells. EM1 cells were treated for 24 h with non-targeting control (Cont), EPAC2, or CALR siRNA and then cultured for 72 h. (A) EM1 cells were stained with SA-β-Gal. Scale bars = 100 μm. (B) The graph shows the relative levels of SA-β-Gal-positive cells. The results from three independent experiments are presented as ratios. (C, D and E) Cell lysates were subjected to immunoblot analysis with an anti-p21, p53, EPAC2, or CALR antibody. The same blot was stripped and re-probed with anti-GAPDH antibody, which served as a loading control (C). The graphs show the relative levels of p21 (D) or p53 (E) normalized to the level of GAPDH. The results (mean ± S.E.M.) from three independent experiments are presented as ratios. **P < 0.01, *P < 0.05 versus Cont.
been reported that the expression of the LIF gene with the p53-binding site in its upstream region is reduced in p53-gene-ablated mice and that reduced expression of p53 leads to infertility via a decrease in the expression of LIF in mice and humans (Hu et al. 2007, Kang et al. 2009). It has also been demonstrated that expression of p53 is dependent on the level of expression of CALR in heterozygous and homozygous CALR-deficient embryos (Mesaiei & Phillipson 2004) and that CALR is abundantly expressed in the implantation site of primate endometrial glands compared with the non-implantation site (Parmar et al. 2009b). Our findings imply that the reduction in the expression of LIF could have resulted in part from the decrease in the expression of p53 in EPAC2- or CALR-silenced EM1 cells.

The results of this study also indicated that the knockdown of EPAC2 or CALR induced senescence in EM1 cells. It has been shown that uterine-specific p53-knockout mice exhibit senescence-associated growth restriction with an increase in endometrial SA-β-Gal activity and p21 expression and experience preterm labor (Hirota et al. 2010). On the basis of these findings, it is conceivable that cellular senescence in EPAC2- or CALR-silenced EM1 cells could impair the production of implantation-related factors due in part to the reduction in CALR-mediated expression of p53 or p21. It is possible that EPAC2-mediated expression of CALR could have prevented the senescence of endometrial glands, which in turn supports the interaction between the endometrium and embryo in humans. It is also conceivable that the prevention of cell senescence mediated through CALR signaling could induce functional changes in endometrial glands during the menstrual cycle in humans. Together, these observations support our postulate that endometrial glandular EPAC2 and CALR could be the regulators of endometrial gland development, and possibly maintenance, during the menstrual cycle and, possibly, implantation.

It has been shown that the influx of calcium in uterine epithelial cells triggers adhesiveness of human trophoblast-like cells (Tinel et al. 2000) and that EPAC2 or CALR regulates extracellular or intracellular influx of Ca2+ (Michalak et al. 2009, Zhang et al. 2009). Hence, it is possible that the downregulation of EPAC2 or CALR in EM1 cells could have caused the reduction in the expression of LIF or PTGS2 and secretion of PGE2 through the dysregulation of the intracellular influx of Ca2+. In addition, S100A11, a Ca2+-binding protein that regulates Ca2+ homeostasis, is expressed in human endometrial cells and involved in the expression of LIF and implantation (Liu et al. 2012). Furthermore, the activation of the epithelial Na+ channel triggers an influx of Ca2+ that leads to the upregulation of the expression of PTGS2 and the release of PGE2 via the activation of PKA in mouse endometrial epithelial cells (Ruan et al. 2012). These results indicate that in EPAC2- or CALR-silenced EM1 cells, downregulation of the production of PKA-mediated implantation-related factors could have resulted from the dysregulation of the Ca2+ influx.

In conclusion, we demonstrated that EPAC2 regulated the expression of CALR in endometrial glandular epithelial cells and that the expression of CALR was crucial for the induction of the implantation-related factors, LIF, PTGS2, and PGE2 in the activation of cAMP signaling. Furthermore, EPAC2 and CALR partially regulated the expression of p53 or p21 and maintained the glandular function for the prevention of cellular senescence.

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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