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Calcium extrusion regulatory molecules: differential expression during pregnancy in the porcine uterus

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ABSTRACT

Calcium ions in the uterine endometrium are essential for the establishment and maintenance of pregnancy, but the cellular and molecular mechanisms of calcium ion regulation in the endometrium are not fully understood. Our previous study in pigs found that calcium regulatory molecules, transient receptor potential, vanilloid type 6 and calbindin-D9K, are expressed in the uterine endometrium during the estrous cycle and pregnancy. However, we did not determine the expression of calcium extrusion regulatory molecules, plasma membrane calcium ATPases (ATP2Bs), sodium/calcium exchangers (SLC8As), or potassium-dependent sodium/calcium exchangers (SLC24As), in the uterine endometrium and conceptuses. Thus, in this study we determine whether ATP2Bs, SCL8As, and SLC24As are expressed in the uterine endometrium during the estrous cycle and pregnancy and in conceptuses during early pregnancy. Real-time RT-PCR analysis showed that *ATP2B*s, *SLC8A*s, and *SLC24A*s were expressed in the uterine endometrium in a pregnancy status- and stage-specific manner. Conceptuses during early pregnancy also expressed these molecules. In situ hybridization analysis showed that *ATP2B1*, *SLC8A1*, and *SLC24A4* were localized mainly to luminal and glandular epithelium and stromal cells in the endometrium during pregnancy. These results indicate that calcium extrusion regulatory molecules are expressed in the uterine endometrium during the estrous cycle and pregnancy and in conceptuses during early pregnancy, indicating that calcium extrusion regulatory molecules may play important roles in the establishment and maintenance of pregnancy by regulating calcium ion concentration in the uterine endometrium in pigs.

1. Introduction

Calcium is essential for cell adhesion between the conceptus trophectoderm and the maternal uterine endometrium during the implantation period [1–3]. Calcium acts not only as an intracellular second messenger for cell signaling but also as an activator of cell adhesion molecules, such as integrins, cadherins, and selectins, for ligand binding during the implantation process [4,5]. In pigs, which form a true epitheliochorial-type placenta, cell-to-cell attachment between the conceptus and the maternal uterus is maintained during the entire pregnancy. Thus, the role of calcium ions in cell adhesion is critical for the establishment and maintenance of pregnancy. At the time of conceptus implantation in pigs, secretion of calcium from the uterine endometrium into the uterine lumen is dynamically regulated and related to the morphologic change of conceptuses; calcium content is low when conceptuses are in a spherical shape on day 10 to 11 of pregnancy, then increases with elongation of conceptuses on day 12 of pregnancy, and decreases thereafter [6]. The fact that treatment with estrogen increases endometrial calcium secretion into the uterine lumen [6] suggests that estrogen produced from the conceptus trophectoderm during the implantation period induces the secretion of calcium ions from the endometrium. However, the detailed cellular and molecular mechanisms of calcium action and

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regulation of calcium secretion and absorption in the uterine endometrium are not fully understood.

Expression of calcium regulatory molecules in the uterine endometrium during the estrous cycle and pregnancy has been investigated in several species, including mice [7,8], rats [9,10], humans [11], and pigs [12,13]. We have shown that transient receptor potential, vanilloid type 6 (TRPV6; also called calcium transporter 1, or epithelial calcium channel 2), which is responsible for calcium entry from outside of the cell, and calbindin-D9k (also called S100 calcium binding protein G; S100G), which is responsible for cytosolic calcium movement inside the cell, are expressed in the uterine endometrium during pregnancy and in a stage-specific manner during the estrous cycle in pigs [12,13].

Several molecules are reported to mediate extrusion of calcium ions across the plasma membranes of the cell. These molecules include plasma membrane calcium ATPase (PMCA1; also called ATPase Ca^{2+} transporting plasma membrane, ATP2B), the sodium/calcium exchanger (also called solute carrier family 8, SLC8A), and the potassium-dependent sodium/calcium exchanger (also called solute carrier family 24, SLC24A) families in the small intestine and kidney [14]. In recent studies, it has been shown that ATP2B1 and SLC24A3 are expressed in the uterine endometrium of rodents and humans [10,11,15–17], suggesting that calcium extrusion regulatory molecules are involved in the maintenance of calcium concentration in the uterine endometrium. Secretion of calcium into the uterine lumen and absorption by the endometrium is active in the uterus at the time of implantation, but expression of calcium extrusion regulatory molecules has not been determined in the uterine endometrium in pigs.

Therefore, to initiate the study of cellular molecular mechanisms of calcium action and regulation of calcium secretion and absorption in the uterine endometrium, this study determined whether calcium extrusion regulatory molecules from families ATP2B, SLC8A, and SLC24A were expressed in the uterine endometrium during the estrous cycle and pregnancy and in early-stage conceptuses in pigs.

2. Materials and methods

2.1. Animals and tissue collection

All experimental procedures that involved animals were conducted in accordance with the Guide for Care and Use of Research Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Yonsei University. Sexually mature crossbred female pigs were assigned randomly to either cyclic or pregnant status. Twenty-four gilts underwent hysterectomy on day 12 and 15 of the estrous cycle and day 12, 15, 30, 60, 90, or 114 of pregnancy (n = 3 gilts/day/status). Pregnancy was confirmed by the presence of fetuses or apparently normal conceptuses in uterine flushings. Endometrium dissected from the myometrium was collected from the middle portion of the uterine horn. Endometrial tissues were snap-frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction. For in situ hybridization, cross-sections of endometrium and conceptuses were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h and then embedded in paraffin.

2.2. Total RNA extraction, cloning of porcine calcium extrusion regulatory molecule subtype genes, and RT-PCR

Total RNA was extracted from endometrial tissues and conceptuses with the use of TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA, USA) according to the manufacturer's recommendations. Ribonucleic acid quantity was assessed spectrophotometrically, and the integrity of the RNA was examined by 1% agarose gel electrophoresis.

Two micrograms of total RNA was treated with DNase I (Invitrogen Life Technology) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen Life Technology) to obtain cDNA. The cDNA templates were then diluted 1:4 with sterile water and amplified by PCR with the use of Taq polymerase (Takara Bio, Shiga, Japan) and specific primers that were based on mRNA sequences of calcium extrusion regulatory molecules for real-time RT-PCR or in situ hybridization analyses (Table 1). Polymerase chain reaction conditions were 35 cycles at 94°C for 45 sec, 54°C for 45 sec, and 72°C for 2 min. Polymerase chain reaction products were separated on 2% agarose gel and visualized by ethidium bromide staining. The identity of each amplified PCR product was verified by sequence analysis after cloning into a pCRII vector (Invitrogen Life Technology).

2.3. Real-time quantitative RT-PCR

To analyze levels of mRNA for the *ATP2B*, *SLC8A*, and *SLC24A* families in the uterine endometrium, real-time RT-PCR was performed with the Applied Biosystems StepOnePlus System (Applied Biosystems, Foster City, CA, USA) with the use of the SYBR Green method. Complementary DNA was synthesized from 4 μg of total RNA isolated from different uterine endometrial tissues (total volume of 21 μL), diluted 1:4 with distilled water, and newly synthesized cDNAs were used for PCR. To maximize efficiency, specific primers that were based on *ATP2B*, *SLC8A*, and *SLC24A* families and ribosomal protein 7 (*RPL7*) gene (Table 1) were designed to amplify cDNA. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for amplification of calcium extrusion regulatory molecules. Final reaction volume was 20 μL, including 2 μL of cDNA, 10 μL of 2× premix, 2 μL of each primer, and 4 μL of diethylpyrocarbone (DEPC)-treated double-distilled water. Polymerase chain reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 35 sec. Data were analyzed with Applied Biosystems software. The results were reported as the expression relative to the level detected on day 12 of the estrous cycle after normalization of the transcript amount to the endogenous RPL7 control by the $2^{-\Delta\Delta CT}$ method [18].

2.4. Nonradioactive in situ hybridization

The nonradioactive in situ hybridization procedure was performed as described previously [19] with some modifications. Sections (5-μm thick) were rehydrated through successive baths of xylene, 100% ethanol, 95% ethanol, DEPC-treated water, and DEPC-treated PBS. Tissue sections were permeabilized with DEPC-treated PBS that contained 0.3% Triton X-100. After washing in DEPC-treated PBS,