

Clinical Study

The Small-Conductance Ca^{2+} -Activated Potassium Channel, Subtype SK3, in the Human Myometrium Is Downregulated in Early Stages of Pregnancy

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The human myometrium is mainly relaxed during pregnancy, but, up to term, contractions become more coordinated and forceful in order to initiate delivery. Small conductance Ca^{2+} -activated K^+ channels (SK channels) in human myometrium have been shown to be downregulated in late pregnancy. The aim was to investigate the presence of SK2 and SK3 in the human myometrium from nonpregnant women, pregnant women at term, and pregnancies delivered preterm and, in addition, to characterize the time of downregulation of these channels. Using qRT-PCR, we observed significantly lower levels of mRNA for SK2 than for SK3 in the nonpregnant tissue. The mRNA levels of SK3 were significantly reduced in tissue from pregnancies at term and pregnancies resulting in preterm deliveries, whereas no downregulation for SK2 was observed. Western blotting confirmed the qRT-PCR results. Using immunohistochemical staining, both SK2 and SK3 were detected in endometrial glandular epithelium. We conclude that SK3 mRNA is downregulated early in pregnancy—at least among those that result in preterm deliveries. Furthermore, we find that SK channels are expressed not only in the uterine smooth muscle but also in the endometrial epithelium.

1. Introduction

Preterm births (delivery before 37 weeks of gestation) account for 9.6% of all births worldwide [1], and the number appears to be rising [2]. Death of the infant due to a preterm birth accounts for 28% of all neonatal deaths globally [3] which makes the issue of a great importance. Treatment available at present is able to delay birth only by approximately 1–2 days, which most often is insufficient to avoid severe complications for the newborn. Thus, there is a need for new and more efficient methods for treatment, calling for improved knowledge regarding the factors contributing to premature delivery.

Recently, focus has been on the K^+ channels and their contribution to the quiescence of the myometrium during pregnancy. Through their role in hyperpolarization, the channels are able to modulate excitability and contractility of the muscle cell. Several potassium channels including KCNQ channels [4], ATP-sensitive potassium channels [5], stretch-activated two-pore potassium channels [6], and big conductance Ca^{2+} -activated potassium channels [7, 8] have been identified in human myometrium, and most of these channels are downregulated during late pregnancy.

The small conductance Ca^{2+} -activated potassium channel (SK channels) comprise three members (SK1–3) [9]. SK3

TABLE 1: Clinical data of the women participating in the study.

Groups of women	Gestational age (weeks)	Maternal age (mean)	Operation	Reason for operations
Nonpregnant (NP)		44 (33–51)	Hysterectomy	Menorrhagia, metrorrhagia, dysfunctional uterine bleeding, and enlarged uterus
Pregnant with preterm delivery (PT)	27–32	27 (21–36)	Acute caesarean section	Intrauterine growth restrictions, contractions, changes in cardiac sound, and complicated pregnancies
Pregnant with delivery at term (not labouring) (TNL)	37–40	34 (22–46)	Elective caesarean section	Disproportion and lack of progress during labour
Pregnant with delivery at term (laboring) (TL)	37–40	30 (27–34)	Acute caesarean section	Breech presentation, maternal request, previous caesarean section, and gemelli

has been described in mouse myometrium of genetically altered mice, where an overexpression depresses phasic uterine contractions and hinders normal parturition [10–12]. In the human myometrium, SK3 has been shown to be downregulated in pregnancies at term both at mRNA level and protein level [13–15]. The presence of SK2 mRNA has also been confirmed in the human myometrium, and one study reports a downregulation at mRNA level in pregnancy [13]. However, no studies have investigated the presence and potential regulation of SK2 protein in humans during pregnancy.

Studies in mice and rats have localized SK3 to the myometrium [11, 16], and one study in mice describes SK3 in the plasma membrane of the smooth muscle cells [17]. Yet, a recent study on human myometrium has shown that SK3 channels are exclusively found in telocytes, an interstitial Cajal-like cell type [18]. A previous study has established the presence of SK3 in immortalized porcine endometrial gland epithelial cells [19]. Taken together, these observations suggest that SK2 and SK3 channels are present both in myometrium and endometrium, but, to date, no studies have confirmed these findings in the human endometrium.

The purpose of the present study was to investigate the expression pattern of SK3 in human myometrium from pregnant women at term and from preterm deliveries. Furthermore, we explored the expression of SK2 in human myometrium from nonpregnant and pregnant women at mRNA and protein level. Finally, we sought to clarify, whether SK2 and SK3 were present in endometrial epithelium.

2. Materials and Methods

2.1. Patients. Human tissue was obtained from pregnant women at elective or acute Caesarean sections. The dominant reason for Caesarean section at term was maternal request, while complicated pregnancies, labour, and preeclampsia were reasons for Caesarean sections in the women with preterm deliveries. Tissue comprising a nonpregnant group was obtained at hysterectomy. At Caesarean section, biopsies were collected from the upper edge of the incision in the lower

uterine segment. Biopsies taken by hysterectomy were from the same area of the uterus, and phenotypically normal tissue was collected. The patients were divided into four groups: (1) nonpregnant women (NP, $n = 14$), (2) women with preterm deliveries before week 32 (PT, $n = 5$), (3) women with deliveries at term but not labouring at the time of surgery (TNL, $n = 18$), and (4) women with deliveries at term where births had been initiated at the time of surgery (TL, $n = 3$). Clinical data of patients are shown in Table 1. All patients gave their full written consent. The project was in accordance with the Helsinki II Declaration and approved by the local Ethics Committee. Tissue for RNA extraction was immediately stabilised in RNAlater (Qiagen, Copenhagen, Denmark). Tissue for protein extraction was transported to the laboratory in the ice-cold Tyrodes buffer and placed at -80°C .

Ideally, all experiments would be made on the same group of patients. However, this was not possible due to the small sizes of the biopsies, and, for this reason, the total number of patients used is greater than the actual numbers in the different analysis.

2.2. RNA Extraction and qRT-PCR. The RNAlater-stabilized tissue was homogenized, and total RNA was extracted (RNAeasy Mini Kit, Qiagen, Copenhagen, Denmark). DNase treatment was used during the extraction as recommended by the manufacturer. The concentration of RNA was measured by spectrophotometry. Total RNA was reversed transcribed with AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, AH Diagnostics A/S Aarhus Denmark) using poly dT primers. cDNA was amplified with Lightcycler 480 SYBr green 1 master mix (Roche Applied Science, Hvidovre, Denmark) or Brilliant II Sybr Green qPCR Mastermix (Agilent Technologies, AH Diagnostics A/S, Aarhus, Denmark). Specific primers were designed in Primer3 [19] with the following sequences: GGAGCAGAG-GAAACTGAATGAC, CAGGGTAAACAATCCTCTTCTCG (SK2 forward and reverse), GACCAAGCCAACACTCTGG, CCAATCTGCTTCTCCAGGTC (SK3 forward and reverse), ACTCTTCCAGCCTTCCTTCC, and AGCACTGTGTTG-CGGTACAG (β -actin forward and reverse). The expected

size of the amplified products was 130, 104, and 117 bp for SK2, SK3, and β -actin, respectively. The primers were designed to include an exon-exon span in the product. Appropriate efficiencies were ensured using standard curves, and samples were run in triplicate. No-template controls and no-reverse-transcription controls were run in parallel. Relative quantification was made by calculating the ratio of target gene to β -actin.

2.3. Protein Extraction and Western Blotting. Protein extraction was performed as previously described [20] with some modifications. Frozen tissue was homogenized in ice cold Tris-EDTA buffer (50 mM Tris, 1 mM EDTA, pH 7.4, added protease inhibitor cocktail (Roche Diagnostics, Hvidovre, Denmark)). After centrifugation (16,000 \times g, 20 min, 4°C), the supernatant was discarded, and the pellet was dissolved in SDS buffer (125 mM Tris-HCl, 4% SDS, 10% glycerol, pH 6.8, added protease inhibitor cocktail) and centrifuged (16,000 \times g, 90 min, 4°C). Protein concentration was measured using the Bradford method (Bio-Rad, Copenhagen, Denmark) using BSA as standards. Proteins were separated on a 4%–12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in a 5% skimmed milk in TBS and 0.05% Tween 20 followed by incubation with the primary antibody (anti-SK2 and anti-SK3 (1:200) Alomone Labs, Jerusalem, Israel [21, 22]) over night at 4°C and with HRP-conjugated secondary antibody (goat anti-rabbit IgG (H + L)-HRP conjugate (1:20,000), Biorad, Copenhagen, Denmark) for one hour at room temperature. Membranes were developed using Super Signal West Pro Chemiluminescent Substrate (Thermo Fisher Scientific, Slangerup, Denmark), and specific protein binding was visualized with chemiluminescence. β -actin was used as reference to confirm equal amounts of protein loaded in each well (Monoclonal Anti- β -actin (1:200) (Santa Cruz Laboratories by AH Diagnostics, Aarhus, Denmark) and EIA grade affinity purified goat anti-mouse IgG (H + L)-HRP conjugate (1:20,000) (Biorad Copenhagen, Denmark)). Intensity measurements of the blots were performed using UN-SCAN-IT gel version 6.1. Samples were normalized to the measured intensity for β -actin in the same sample.

2.4. Immunohistochemistry. Slides containing sections of paraffin embedded human endometrial cells from cervical scraping obtained by papanicolaou smear tests were nicely provided by Steen Seier Poulsen. The sections were deparaffinized in xylene/ethanol series. Following antigen retrieval in citric acid for 15 min in microwave oven, the sections were blocked by PBS added 2% BSA and incubated with the primary antibody over night at 4°C (rabbit anti-SK3 (1:2000) in PBS added 2% BSA, Alomone Labs, Jerusalem, Israel). After wash with PBS, the sections were incubated with the secondary antibody for 40 min at room temperature (biotinylated goat anti-rabbit IgG (H + L) from Vector Laboratories, by VWR Bie & Berntsen, Rodovre, Denmark). Subsequently, the sections were washed and incubated in streptABComplex/horseradish peroxidase (Dako, Glostrup, Denmark) for 30 min, followed by incubation with 3,3'-Diaminobenzidine for 15 min. Finally, counterstaining was

performed with hematoxylin, and the sections were dehydrated with ethanol and mounted with cover glass.

2.5. Statistics. qRT-PCR data was analysed using GraphPad Prism Version 5.03 (GraphPad Software, San Diego, California, USA). Comparison of mRNA expression for SK2 in pregnant versus nonpregnant and the western blot data for SK2 and SK3 were performed using an unpaired two-tailed *t*-test. mRNA expression for SK3 was performed using one-way analysis of variance with Bonferroni's multiple comparison test. $P < 0.05$ was considered significant (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

3. Results

3.1. SK2 and SK3 in Human Myometrium during Pregnancy. To investigate mRNA level for SK2 and SK3 during pregnancy, total RNA was extracted from myometrial tissue from nonpregnant women and pregnant women at term, and qRT-PCR was conducted. The ratio of SK2 mRNA relative to that of SK3 in tissue from nonpregnant women was 0.092 ($n = 6$), which indicated a higher level of SK3 than SK2 (10.9 times) in the nonpregnant human myometrium.

To explore any changes in the mRNA levels for SK2 during pregnancy, a comparison was made between tissues from nonpregnant and pregnant women at term (not labouring) (Figure 1(a)). No significant difference was observed between the two groups indicating that SK2 does not change at the mRNA level during pregnancy. This finding was confirmed at the protein level by Western blotting (Figures 1(b) and 1(c)).

In contrast to the mRNA level of SK2, the mRNA level of SK3 changed during pregnancy (Figure 2(a)). A significantly lower level of mRNA was observed in the myometrium from pregnant women compared to the nonpregnant group. This was observed both for the preterm group as well as the pregnant group at term, nonlabouring and labouring. No significant difference between the pregnant women was seen. The downregulation of SK3 mRNA seen during pregnancy was confirmed at protein level in the tissue from nonlabouring pregnant women at term (Figures 2(b) and 2(c)).

3.2. Localization of SK2 and SK3 in Endometrial Epithelium. To investigate the localization of SK2 and SK3 in the human endometrium, slides containing endometrial cells from cervical scraping were stained with SK2 and SK3 specific antibodies. Results of the staining are presented in Figure 3. Specific signals for both SK2 and SK3 were detected in the glandular epithelium both in scraping from the proliferative phase (Figures 3(a) and 3(d) for SK2 and SK3, resp.) and from the secretory phase (Figures 3(b) and 3(c) for SK2 and Figures 3(e) and 3(f) for SK3, resp.). Weak staining for SK2 and SK3 was also observed in the stromal cells.

4. Discussion

In the present study, we investigated the expression of SK2 and SK3 in the human nonpregnant and pregnant myometrium. SK3 was found to be the predominant of these

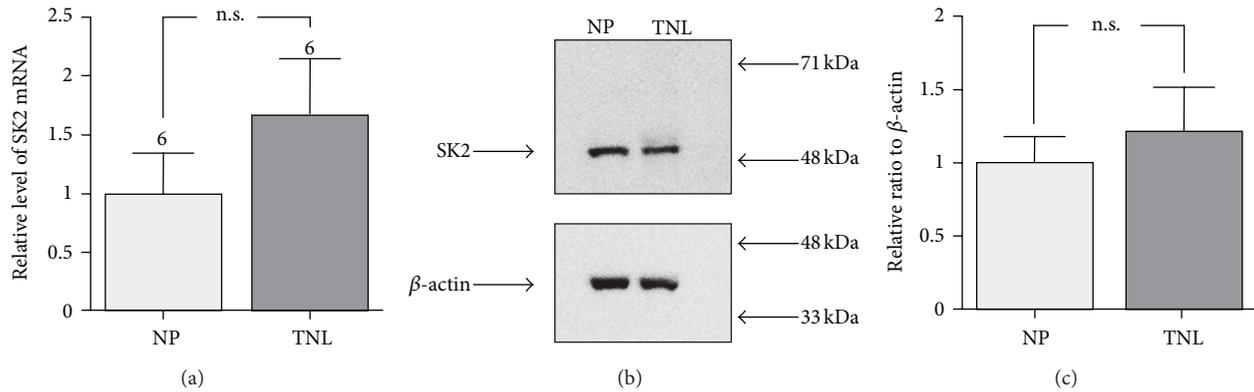


FIGURE 1: The mRNA level of SK2 in nonpregnant (NP) and pregnant women at term not labouring (TNL) expressed relative to β -actin (a). Data is presented as mean \pm S.E.M. (1 ± 0.34 and 1.67 ± 0.47 for NP and TNL, resp.). No significant downregulation was observed in the pregnant samples. ($n = 6$ and $n = 6$ for NP and T, resp.). Protein expression of SK2 in nonpregnant (NP) and pregnant to term (TNL) (b). Blots show the expected molecular weight of ~ 57 kDa and ~ 43 kDa for SK2 and β -actin, respectively ($n = 3$ for both groups). (c) Accumulated measurements of protein expression from both groups of patients. All data are ratio to β -actin in the same sample. Data is presented as mean \pm SEM (1 ± 0.19 and 1.21 ± 0.30 for NP and TNL, resp.).

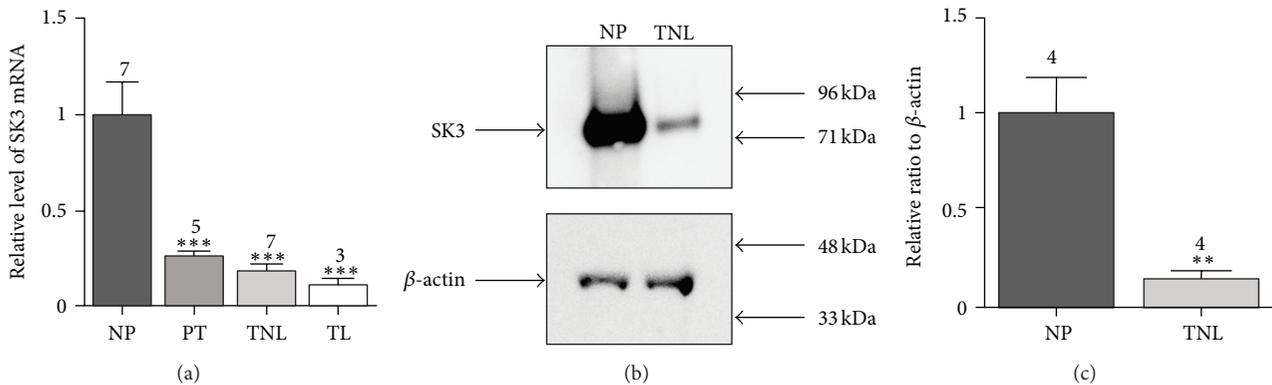


FIGURE 2: The mRNA levels of SK3 in nonpregnant women (NP), preterm deliveries (PT), pregnancy at term not laboring (TNL), and pregnancy at term laboring (TL) expressed relative to β -actin, (a). Data is presented as mean \pm SEM (1 ± 0.17 ($n = 7$), 0.27 ± 0.022 ($n = 5$), 0.19 ± 0.028 ($n = 7$), and 0.11 ± 0.027 ($n = 3$) for NP, PT, TNL, and TL, resp.). *** $P < 0.001$ (versus NP). Protein expression of SK3 in nonpregnant (NP) and pregnant at term not laboring (TNL) (b). Blots show the expected molecular weight of ~ 70 kDa and ~ 43 kDa for SK3 and β -actin respectively ($n = 4$ for both groups). (c) Accumulated measurements of protein expression from both groups of patients. All data are ratio to β -actin in the same sample. Data is presented as mean \pm SEM. (1 ± 0.18 and 0.14 ± 0.041 for NP and TNL, resp.). Asterisk (**) represents significant difference from NP patients ($P < 0.01$).

two channels in the nonpregnant tissue at the mRNA level. The mRNA level of SK2 was negligible compared to that of SK3. No downregulation of SK2 appeared to take place during pregnancy, neither at the mRNA nor at the protein level. In contrast, the level of SK3 mRNA was significantly lower in tissue from the three groups of pregnant women. Beside their presence in myometrial tissue shown at mRNA and protein level, both SK2 and SK3 were localized to the endometrial glandular epithelium.

The expression of SK2 in the human myometrium has been investigated only scarcely, as most interest has been on SK3. However, one study reports the presence and downregulation of SK2 mRNA in human myometrium during pregnancy [13]. In the present study, we found no change in

the mRNA level for SK2 during pregnancy, and this result was confirmed at protein level. The discrepancy between our study and the one by Mazzone et al. is possibly a result of a small amount of SK2 mRNA present in the myometrium, which may cause uncertainties in the qPCR technique. Indeed, we found the amount of SK2 mRNA to be insignificant compared to that of SK3, and, therefore, we conclude that SK2 expression does not seem to be important in the human myometrium during pregnancy.

The observed downregulation of SK3 in human myometrium during pregnancy seen in the present study is supported by several other studies [13, 15, 18]. The stable mRNA level in the nonlabouring and laboring women in pregnancies at term indicates that the expression of SK3

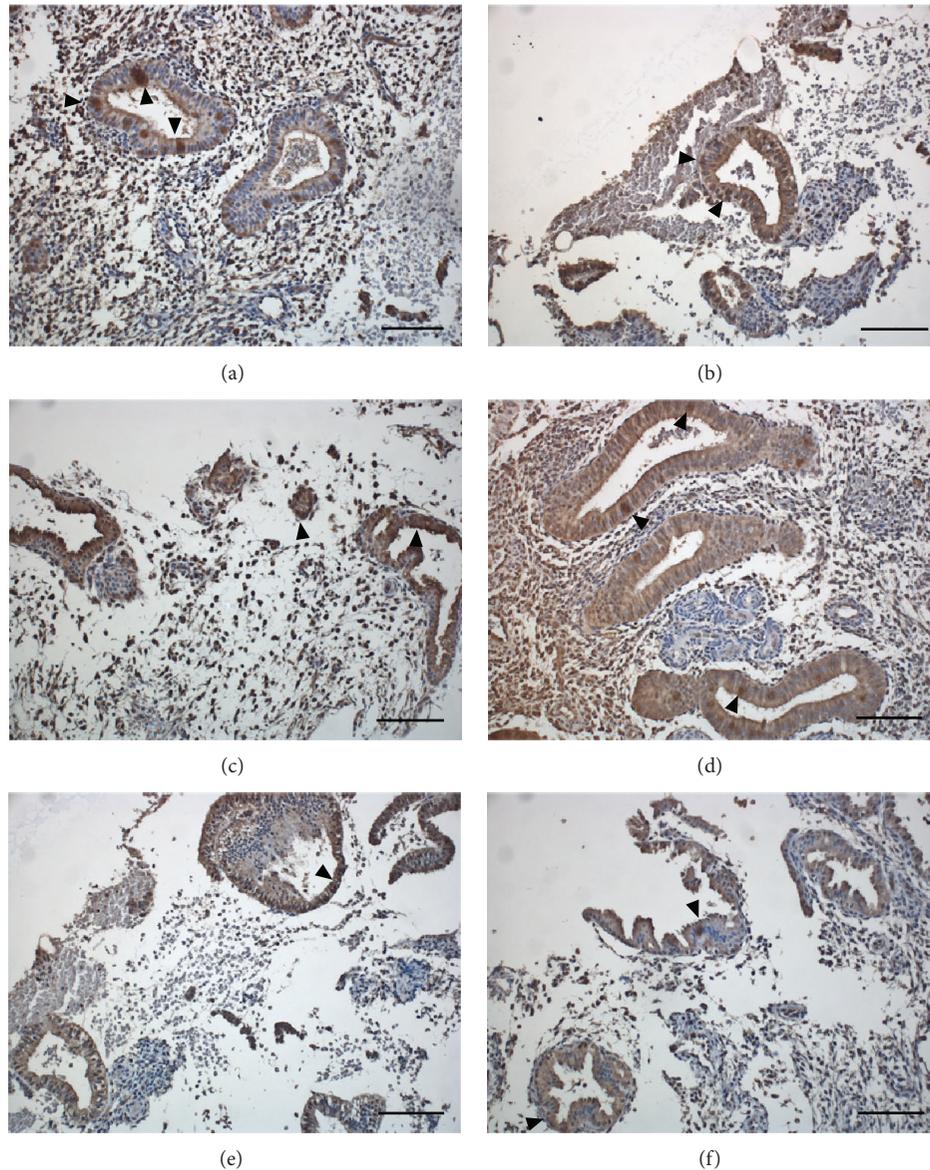


FIGURE 3: Human endometrial cells from cervical scraping stained for SK2 and SK3. SK2 staining in samples taken from proliferative phase (a) and secretory phase ((b) and (c)) of menstrual cycle. SK3 staining in samples also from proliferative phase (d) and secretory phase ((e) and (f)). All pictures are presented in 20x magnification, and all scale bars represent 0.1 mm. Arrow heads point at epithelial location.

mRNA is not directly linked to contractions of the uterus. This finding is supported by a similar finding in the study by Pierce and England [15].

To the authors' knowledge, the present study is the first to report the downregulation of the channel in pregnancies as early as gestational week 27–32. Though, the number of patients in the group of pregnant women with preterm deliveries was limited, the SEM value observed in the group was very small, which strengthens the finding.

Studies in mice have reported a downregulation of SK3 mRNA early in pregnancy [12, 17], which implies that the downregulation observed in our study could be the general scheme. Whether this is the case or the early

downregulation is related to the pathological pregnancies is currently unknown and out of the scope of this study.

Several studies have examined the localization of SK3 in the myometrium, and SK3 has been localized to the smooth muscle layer in mice and rats [11, 16] and the cell membrane of the smooth muscle cell in mice [17]. Recently, our lab described the SK3 channels in the myometrium of nonpregnant and women pregnant at term nonlabouring, and localized these to the telocytes in human myometrium [18]. However, no studies have explored the presence of the channels in the endometrium, despite the fact that the channels might also play a role in this layer of the uterus. As we have previously identified SK2 and SK3 in the rat

endometrial glandular and luminal epithelium (unpublished observation), it was tempting to investigate if this localization could be found in human uterus as well. Indeed, specific staining both for SK2 and SK3 was detected in the glandular epithelium obtained from cervical scraping taken from both the proliferative and the secretory phase. Despite the difference in the structure of the endometrium during the menstrual cycle, no obvious variation in the staining pattern for SK3 was observed in the proliferative and secretory phase.

The levels of the steroid hormones estrogen and progesterone vary throughout the human menstrual cycle, with an estrogen peak in late proliferative phase and a lower peak in the secretory phase [23]. The research has clarified an effect of 17β -estradiol on the SK3 expression level [24], so, for this reason, we would expect to see a difference in the proliferative and secretory phase reflecting the hormonal fluctuations. However, a recent study investigating the effect of 17β -estradiol on the SK3 expression in mouse uterus [15] observed no change in the expression pattern of SK3 in the mouse uterus after hormonal treatment, which strengthens our findings.

The localization of SK2 and SK3 in the epithelium points to a new role of these channels in the uterus in addition to their contribution to the quiescence of the myometrium.

L-type Ca^{2+} channels have been identified in the endometrial epithelium of mice [25], where they mediate a Ca^{2+} -influx following depolarization of the epithelial cell. This depolarization is caused by activation of Na^{+} channels that also present in the epithelial membrane [25]. It is easy to imagine the activation of SK channels as a response to the elevated Ca^{2+} concentration in the epithelial cell. A possible role of SK channels in the endometrial epithelium is repolarization and restoration of the resting membrane potential. Several K^{+} channels have been identified in other epithelial tissues such as the kidney, where they play a central role for the maintenance of the membrane potential [26]. Supported by this, we suggest that SK2 and SK3, in addition to the contribution of SK3 in the quiescence of the myometrium during pregnancy, contribute to maintenance of the membrane potential in the endometrial epithelial cells.

In summary, we have found the expression of SK2 in human myometrium, where it is constantly expressed throughout pregnancy. Furthermore, we have shown that the downregulation of SK3 mRNA in the myometrium takes place early in pregnancy at least in women with pathological pregnancies. And, lastly, we have identified both SK2 and SK3 in the endometrial epithelium, which suggest multiple functions of the SK channels in the uterus.

Abbreviations

NP: Nonpregnant
 PT: Preterm deliveries
 TNL: Pregnancies at term not labouring
 TL: Pregnancies at term labouring.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgments

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