Differential expression of uterine calcium transporter 1 and plasma membrane Ca\(^{2+}\) ATPase 1b during rat estrous cycle

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Differential expression of uterine calcium transporter 1 and plasma membrane Ca\(^{2+}\) ATPase 1b during rat estrous cycle. *Am J Physiol Endocrinol Metab* 292: E234–E241, 2006; doi:10.1152/ajpendo.00434.2005.—Calcium-related proteins include the calcium transporters 1 and 2 (CaT1 and CaT2), plasma membrane Ca\(^{2+}\)-ATPase 1b (PMCA1b), and calbindin-D9k and -D28k. The expression of CaT1 and PMCA1b and their potential roles in the uterine tissue remain to be clarified. Thus, in the present study, the expression patterns of CaT1 and PMCA1b were examined to predict their roles in rat uterus during the estrous cycle. Both CaT1 and PMCA1b mRNAs were detected in rat uterus. Uterine CaT1 mRNA was highly expressed at diestrus compared with proestrus, whereas PMCA1b expression was not altered during the estrus cycle. To evaluate the sex steroids involved in uterine CaT1 mRNA regulation, 17β-estradiol (E2) and/or progesterone (P4) were injected into immature rats. Treatment with P4 or E2 plus P4 resulted in an increase in CaT1 mRNA, but a synergetic effect of E2 plus P4 was not detected. Uterine CaT1 mRNA was induced by P4 in a time- and dose-dependent manner, with maximal transcript detected 12 h after the final P4 injection. Treatment with RU486, a progesterone receptor (PR) antagonist, completely blocked P4-induced CaT1 mRNA, indicating that P4 regulates CaT1 mRNA expression via a PR-mediated pathway. In addition, CaT1 mRNA was expressed in uterine endometrium and glandular endometrium at diestrus in P4-treated rats. Together, these results suggest that CaT1 is regulated by P4 at diestrus via a PR-dependent pathway.

transient receptor potential vanilloid 6; uterus

THE ACTIONS OF CALCIUM IONS IN FEMALE REPRODUCTIVE ORGANS have been widely studied for several decades. It has been suggested that calcium ions are involved in uterine smooth muscle contraction and implantation. The balance between uterine contraction and relaxation is extremely important throughout pregnancy and during labor. However, the regulation and mechanism of uterine calcium are not fully understood. The control of excitability and contraction in the myometrium is important for reducing fetal and maternal mortality caused by pre- and postmature birth or metaplasia (36). In addition, calcium action on the endometrium is important for implantation. Several kinds of the calcium-processing proteins are expressed in the uterus, including calcium-channel proteins that mediate muscle contraction and implantation (24).

The major roles of calcium-related proteins are calcium influx, transfer through, and extrusion out of the cell membrane. These steps are carried out by the calcium entry channel proteins of the outer membrane, cytosolic buffering proteins, and the excreting pump proteins in the intestinal model (34). Two epithelial calcium channels, calcium transporters 1 (CaT1) and 2 (CaT2), found in the apical membranes of intestinal and renal epithelial cells, were proposed to be mediators of calcium uptake during transcellular calcium transport (4). CaT1 is also designated as epithelial calcium channel 2 (EcaC2) or transient receptor potential vanilloid (TRPV6), and CaT2 is also known as EcaC1 or TRPV5. These closely related proteins are primarily involved in calcium absorption or reabsorption in various cell types of the duodenum and kidney (28). CaT1 was first cloned from the rat duodenum and detected in human and mouse duodena (2, 21). CaT2, induced by the hormone-form of vitamin D, is expressed in rabbit renal epithelial cells and has been detected in human, rat, and mouse kidneys. The CaT1 and CaT2 genes are juxtaposed on the same chromosome, and their genomic structures are similar (18, 19, 35). CaT1 is distributed in the duodenum, jejunum, ileum, and kidney and in excocrine tissues such as the pancreas, prostate, and mammary and sweat glands (10, 19, 20, 35, 37), whereas CaT2 is only expressed in the kidney. In reproductive organs, CaT1 is expressed in the placenta and uterine muscle. Placental CaT1 plays a role as a calcium transporter to the fetus (16, 17). Duodenal and renal CaT1 expression is regulated by vitamin D, estrogen, and dietary calcium ion. An active form of vitamin D increases duodenal calcium absorption, and abnormal calcium absorption has been observed in a vitamin D-receptor knockout mouse (7, 8). Dietary calcium also induces duodenal and renal CaT1 mRNA expression (33). In addition, estrogen therapy for menopausal women induces duodenal CaT1 mRNA, suggesting that estrogen independently modulates CaT1 expression (32).

Plasma membrane Ca\(^{2+}\) ATPase (PMCA) removes calcium from the cytosol to the extracellular fluid. PMCA located in cellular membranes acts as a calcium eliminator, using an ATP-dependent pump. PMCA, first detected in erythrocyte membranes, has a high affinity for calcium ion (25). In many species, PMCA is involved in calcium homeostasis (25, 26, 29). PMCA has four isoforms (PMCA1–4), which are further divided into several subtypes by alternative splicing. PMCA1 is known as the housekeeping isoform, because its mRNA is found in all...
tissues. However, PMCA1b increases in uterine smooth muscle during labor (30), and the placental PMCA1b acts as a calcium transporter with CaT1. Although the factors involved in the regulation of PMCA1b are not clear, vitamin D and estrogen putative regulators in the uterus (34). PMCA1s are distributed in a tissue-specific manner, and various patterns of expression are observed during the menstrual cycle and pregnancy (30).

Because of the major function of calcium-related proteins in the uterus, this study was performed to clarify how the expression of uterine CaT1 and PMCA1 might be regulated by 17β-estradiol (E2) and/or progesterone (P4) and to determine the spatial expression of CaT1 mRNA in the rat uterus.

MATERIALS AND METHODS

Animals and treatments. Mature (>14 wk old) and immature (14 day old) female Sprague-Dawley rats were obtained from Dae Han Biolink (Eumseong, Chungbuk, Korea). All animals were housed in polycarbonate cages and used after acclimation to an environmentally controlled room (temperature, 23 ± 2°C; relative humidity, 50 ± 10%; frequent ventilation, 12-h light cycle). Mature rats (total n = 12) were divided into three groups (proestrus, diestrus, and estrus) by vaginal cell morphology. Bilateral ovariectomized (OVX) rats were given subcutaneous injections of P4 at 1, 4, 5, and 10 mg/kg body wt as a positive control, and a second group (n = 12) consisted of the vehicle (ethanol alone as a negative control). To rule out the possible involvement of the glucocorticoids receptor (26), dexamethasone (Dex; 0.1, 0.75, and 4 mg/kg body wt) for 3 days and euthanized 12 h after the final injection. Two groups of OVX rats (total n = 6) were injected subcutaneously with ethanol or 4 mg/kg body wt P4 (Sigma-Aldrich) daily for 3 days and euthanized 12 h after the final injection. In the time-dependent experiment, 24 rats were injected subcutaneously with P4 (4 mg/kg body wt) for 3 days and euthanized 12 h after the final injection. In the dose-dependent experiment, five groups of five rats were given subcutaneous injections of P4 at 1, 4, 5, and 10 mg/kg body wt for 3 days and euthanized 12 h after the final injection. Three groups of rats (total n = 9) were injected subcutaneously with 0.4, 4, and 40 mg/kg body wt RU486 30 min before injection with P4 (4 mg/kg body wt) for 3 days. Another group (n = 3) received 4 mg/kg body wt P4 as a positive control, and a second group (n = 3) received ethanol alone as a negative control. To rule out the possible involvement of the glucocorticoids receptor (26), dexamethasone (Dex; 0.1, 0.5, or 1 mg/kg body wt) was injected into rats (n = 9). All experimental procedures and animal use were approved by the Ethics Committee of the Chungbuk National University.

Total RNA extraction and semiquantitative RT-PCR. Rats were euthanized, and uteri were rapidly excised and washed in cold, sterile NaCl (0.9%). Total RNA was prepared from the uteri with TRIzol reagent (Invitrogen, Carlsbad, CA), and the concentration of RNA was determined by absorbance at 260 nm. RT-PCR was performed as previously described (11). Briefly, total RNA (1 μg) was reverse transcribed into first-strand cDNAs using Moloney murine leukemia virus reverse transcriptase (iNtRON Bio, Sungnam, Kyungki-Do, Korea) and random primers (9-mers; TaKaRa Bio, Otsu, Shiga, Japan). CaT1 and IA (a housekeeping gene) were amplified using 25 and 18 cycles, respectively. The cDNAs were amplified in a 20-μl PCR reaction containing 1 unit of Taq DNA polymerase (iNtRON), 1.5 mM MgCl2, 2 mM dNTPs, and 50 pmol of specific primers. PCR reactions were denatured at 95°C for 1 min, annealed at 63°C for 1 min, and extended at 72°C for 90 s. The oligonucleotide primers for CaT1 were 5′-AGG ATC TGG GAG TCG CCT CTT-3′ (sense) and 5′-GGG GGT CTT TGG CTC ATG TGT CAT-3′ (antisense). PCR amplification using the Smart Cycle system (TaKaRa Bio) began with an initial denaturation at 95°C for 30 s. Each of the 35 amplification cycles consisted of denaturation at 95°C for 5 s, annealing at 55°C for 7 s, and extension at 72°C for 12 s. Relative expression levels for each sample were calculated based on the cycle threshold (Ct) and monitored amplification curve. PCR amplification curves were evaluated by fluorescence of the double-stranded DNA-specific dye SYBR green I versus the amount of standard PCR product. The expression of CaT1 was normalized to IA mRNA.

In situ hybridization. A 333-bp rat CaT1 cDNA fragment was reverse transcribed and amplified from total RNA with the use of the forward primer 5′-AGG ATC TGG GAG TCG CCT CTT-3′ and reverse primer 5′-GTG GGA GAC AGA GCC GCT AGC-3′, designed to amplify bp 419–751 of rat CaT1 (GenBank accession no. NM035368). The amplification of CaT1 cDNA was carried out for 27 cycles at 95°C for 1 min, 63°C for 1 min, and 72°C for 90 s. The CaT1 amplification product was purified and cloned into the pcDNA3 vector (5.4 kb; Invitrogen). Digoxigenin-labeled antisense and sense cRNA probes were transcribed in vitro using a digoxigenin RNA labeling kit (IT7 for sense and SP6 for antisense; Roche, Mannheim, Germany). Uterine tissues were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin according to standard laboratory techniques. Paraffin blocks were cut into 6-μm sections and mounted on poly-l-lysinated slides. The sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol, diethyl pyrocarbonate (DEPC)-treated water, and DEPC-treated PBS (twice for 5 min each). The slides were postfixed for 10 min in 4% paraformaldehyde-PBS, incubated twice for 15 min in PBS containing 0.1% active DEPC, and equilibrated for 15 min in 5× SSC (0.75 M NaCl, 0.075 M Na-citrate). The sections were prehybridized for 2 h at 58°C in the hybridization mix (50% formamide, 5× SSC, 0.2 g/ml dextran sulfate, 10 mg/ml polyA, 10 mg/ml salmon sperm DNA, 10 mg/ml yeast tRNA, 0.1 ml/ml 1 M DTT, and 10 μl/ml 50× Denhardt’s solution). The probes were denatured for 5 min at 80°C and added to the hybridization mix (400 ng/ml). The hybridization reaction was carried out at 58°C for 24–48 h. The hybridized sections were washed for 30 min in 2× SSC (room temperature), 1 h in 2× SSC (65°C), and 1 h in 0.1× SSC (65°C) and equilibrated for 5 min in buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.5). The sections were then incubated for 2 h at room temperature with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche), diluted 1:2,000 in buffer 1 containing 1× blocking reagent (Roche). The signal was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche) in buffer 2.

Data analysis. Data were analyzed using the nonparametric one-way analysis of variance with the Kruskal Wallis test, followed by Dunnnett’s test for multiple comparisons to vehicle. The data values were converted to ranks for these tests. All statistical analyses were performed with SPSS for Windows (SPSS, Chicago, IL). P < 0.05 was considered statistically significant.

RESULTS

Expression of uterine CaT1 and PMCA1 mRNAs during estrous cycle. To investigate the expression of uterine CaT1 and PMCA1 during the estrous cycle, we divided mature rats
Fig. 1. Uterine calcium transporter 1 (CaT1) and plasma membrane Ca\(^{2+}\)-ATPase (PMCA1) mRNA expression in adult female rats during the estrous cycle. Rats were divided into three groups (proestrus, estrus, and diestrus) according to vaginal cell morphology. As shown in Fig. 1A, CaT1 mRNA expression was increased 10-fold at diestrus over that at proestrus. However, PMCA1 mRNA was not altered during the cycle (Fig. 1B).

Effect of sex steroid hormones E\(_2\) and P\(_4\) on uterine CaT1 mRNA. To elucidate possible factors involving uterine CaT1 mRNA expression, the sex steroid hormones E\(_2\) and/or P\(_4\) were injected into immature rats. The expression of CaT1 mRNA was detected by RT-PCR and real-time PCR after injection with ethanol as the vehicle, E\(_2\) (40 \(\mu\)g/kg body wt), or P\(_4\) (4 mg/kg body wt) for 3 days. CaT1 mRNA was significantly induced by P\(_4\) (10-fold vs. vehicle) as shown in Fig. 2A, whereas the mRNA level was not altered by E\(_2\), suggesting that P\(_4\) is the dominant secreted hormone during diestrus and induces CaT1 transcripts at this stage. OVX rats were injected with ethanol or P\(_4\) (4 mg/kg body wt) for 3 days and uterine CaT1 expression was measured. A significant increase in CaT1 mRNA was observed (Fig. 2B), indicating that P\(_4\) induces the uterine CaT1 gene in both immature and OVX rats.

Time- and dose-dependent effects of P\(_4\) on expression of uterine CaT1 mRNA. For a time course of CaT1 mRNA induction by P\(_4\), immature rats were injected with P\(_4\) (4 mg/kg body wt) for 3 days, and their uteri were isolated at 3, 6, 12, 24, 48, and 72 h after the final injection. Notably, increased CaT1 mRNA was observed at 6 h and maximal induction was detected at 12 h after the final injection (a 4.5-fold increase over that at 3 h) as shown in Fig. 3. The increased level of CaT1 mRNA expression was maintained until 72 h after the final injection.

To clarify the effect of P\(_4\) on the regulation of CaT1 mRNA in a dose-dependent manner, three different doses of P\(_4\) (0.4, 4, and 40 mg/kg body wt) were administrated to immature rats for 3 days. A significant induction of CaT1 mRNA was observed at all doses of P\(_4\) in a dose-dependent manner (Fig. 4). Although treatment with P\(_4\) (4 mg/kg) significantly increased uterine CaT1 mRNA, no synergic effect of P\(_4\) plus E\(_2\) was observed compared with that of P\(_4\) alone, as shown in Fig. 4.

Effects of PR antagonist RU486 or Dex on uterine CaT1 mRNA induction by P\(_4\). To elucidate whether progesterone receptor (PR) mediation was involved in P\(_4\)-induced uterine CaT1 transcription, we injected RU486 (0.4, 4, and 40 mg/kg body wt), a PR antagonist, into immature rats 30 min before P\(_4\) (4 mg/kg body wt) treatment for 3 days. As shown in Fig. 5A, a physiological dose of P\(_4\) (4 mg/kg body wt) significantly induced uterine expression of CaT1 mRNA. However, pre-treatment with RU486 completely blocked P\(_4\)-induced CaT1 mRNA expression. To rule out any possible involvement of the glucocorticoids receptor (GR) (26), Dex (0.1, 0.5, and 1 mg/kg body wt), a strong GR agonist, was injected into rats (\(n = 9\)). Treatment with Dex did not alter the expression of the CaT1 mRNA (Fig. 5B), suggesting that P\(_4\)-induced CaT1 expression is not partially mediated through the GR in uterine tissue.

Localization of CaT1 expression in rat uterus. To elucidate the spatial expression of uterine CaT1 transcripts during the estrous cycle, we used a specific probe to detect CaT1 mRNA.
in untreated and P₄-treated uteri. Uterine CaT1 mRNA was moderately expressed in the endometrial layer and glandular epithelium at diestrus (Fig. 6A). In parallel with its uterine expression at diestrus, intense positive-stained cells for CaT1 mRNA were observed in the identical endometrial cells and uterine glands of P₄-treated rats (Fig. 6B). No positively staining cells were detected at other stages of the estrous cycle (Fig. 6A).

**DISCUSSION**

The aim of this study was to elucidate the expression and regulation of calcium-related genes in the rat uterus by endogenous or exogenous sex hormones. A number of genes are known to be involved in uterine function during the estrous cycle (3). These include calcium-binding proteins (CaBP-9k and CaBP-28k), monoclonal nonspecific suppressor factor/H9252, and the splicing factor SC35, which are involved in embryo implantation during the “window of receptivity” of the uterine endometrium (14, 23, 27). CaBPs have a high affinity for calcium ions, which are known to be an important factor for implantation and uterine function. However, other calcium-processing proteins also may have specific roles in uterine function.

In this study, we examined whether two calcium transporter proteins (CaT1 and CaT2) and the calcium exclusion protein (PMCA1) are expressed in the uterus and whether their expression is altered during the estrous cycle (proestrus, diestrus, and estrus). RT-PCR confirmed that two genes, CaT1 and PMCA1, were expressed in the uteri of rats. CaT1 mRNA varied during the estrous cycle, whereas PMCA1 did not. In a previous study, the isoforms PMCA1 and PMCA4 were identified as housekeeping genes in the human placenta, and uterine PMCA1 appeared to be normally expressed in the uterus at a constant level (22). The less abundant calcium transporter gene CaT2 was not detected in the uterus of rats in this study, and Weber et al. (35) found that CaT2 was only expressed in the kidney. The CaT1 transcript increased up to 10-fold at diestrus, a P₄-dominant phase of the reproductive cycle, implying that it might be involved in a specific uterine function such as calcium ion processing. The sex steroids E₂ and P₄ are the major hormones that regulate uterine structural change and function, and their levels alternate during the estrous cycle. To elucidate which factors are involved in uterine CaT1 transcription, we injected immature and OVX rats daily with E₂ and/or P₄. A physiological dose of P₄ or P₄ combined with E₂ induced uterine CaT1 mRNA in immature rats, and P₄ alone further increased its expression in the estrogen-deficient (OVX) rats. Although E₂ is the dominant secreted hormone at proestrus, it did not affect CaT1 expression. P₄ plus E₂ induced the same expression levels of CaT1 as P₄ alone. These results imply that P₄ may be a major regulator of uterine CaT1 transcription in untreated and P₄-treated uteri. Uterine CaT1 mRNA was moderately expressed in the endometrial layer and glandular epithelium at diestrus (Fig. 6A). In parallel with its uterine expression at diestrus, intense positive-stained cells for CaT1 mRNA were observed in the identical endometrial cells and uterine glands of P₄-treated rats (Fig. 6B). No positively staining cells were detected at other stages of the estrous cycle (Fig. 6A).
In the time course experiment, uterine CaT1 mRNA was significantly elevated at 12 h after the final P4 injection of a 3-day series and disappeared after 24 h. In addition, CaT1 mRNA expression was stimulated by P4 in a dose-dependent manner. These data suggest that uterine CaT1 transcription is solely dependent on P4 in the rat model.

In contrast to uterine CaT1 mRNA expression, the expression of CaBP-9k, a well-known calcium-regulating gene in the uterus, has been shown to increase at proestrus and is induced by E2 and blocked by P4 in the rat uterus (12). Whereas mouse CaBP-9k knockouts in normal implantation or that CaT1 may compensate for uterine CaBP at different stages.

CaBP, CaT1, and PMCA1 are major components of calcium ion processing in duodenal models, and their function and regulation have been progressively studied in the gastrointestinal track (33). Intestinal CaT1 was highly induced by exogenous E2 treatment, and its induction was blocked by an estrogen receptor antagonist (34). CaBP transcripts were also increased by E2; however, PMCA1 was not altered. Weber et al. (35) reported that the CaT1 gene possessed an estrogen response element (ERE) in its promoter sequence; however, other groups did not find an ERE in the CaT1 gene (9, 33, 34). Although Van Cromphaut et al. (34), using estrogen receptor (ER)-α and -β KO mice, demonstrated that ER-α may be a major pathway in the induction of CaT1 transcription, an exact pathway has not been characterized yet.

**Fig. 5. Effects of RU486 (RU) and dexamethasone (Dex) on P4-induced uterine CaT1 mRNA.** Three groups of rats (total n = 9) were subcutaneously injected daily for 3 days with RU (0.4, 4, or 40 mg/kg body wt) 30 min before injection with 4 mg/kg body wt of P4. The positive control group (n = 3) received 4 mg/kg body wt of P4 alone. The negative control group (n = 3) received ethanol alone. All rats were euthanized 12 h after the final injection. To rule out the possible involvement of the glucocorticoid receptor, Dex (0.1, 0.5, or 1 mg/kg body wt) was injected into rats (n = 9) 30 min before injection with P4 (4 mg/kg body wt). A: gels (top) indicate the effect of RU on P4-induced CaT1 mRNA expression as determined by RT-PCR. Bar graph (bottom) represents the effect of RU on P4-induced CaT1 expression as determined by real-time PCR. B: bar graph indicates the effect of Dex on CaT1 mRNA expression as determined by real-time PCR. Results are means ± SE of duplicate values from all samples, expressed as percentages of CaT1 mRNA. *P < 0.05, significantly different from the vehicle. **P < 0.05, significantly different from the P4-treated group.
Thus estrogens are independent potent regulators of the expression of this calcium influx gene, which is involved in active intestinal calcium absorption (1, 34). To elucidate the exact pathway for the induction of uterine \( \text{CaT1} \) by \( P_4 \), we injected a PR antagonist into immature rats 30 min before \( P_4 \) treatment. \( P_4 \)-induced uterine \( \text{CaT1} \) expression was completely blocked by the PR antagonist. This result suggests that \( P_4 \) activates its receptor to induce uterine \( \text{CaT1} \) transcription, which may be mediated by a PRE or indirectly via stimulation of a transcriptional factor such as activating protein 1 or stimulating protein 1 (5, 27, 31). To rule out the possibility of GR involvement, we injected rats with Dex, a strong GR agonist. It has been shown that Dex binds to the GR 6,900-fold more strongly than to the PR (13). Treatment with Dex did not alter the expression of \( \text{CaT1} \) mRNA in the rat uterus, suggesting that \( P_4 \)-induced \( \text{CaT1} \) expression is not mediated by the GR in this tissue.

\( \text{CaT1} \) has been found to be highly expressed in a number of exocrine organs, including the pancreas, prostate, and mammary gland (10, 19, 20, 35, 37). However, its role in the uterus has not been established. In this study, \( \text{CaT1} \) transcripts were observed in the endothelium layer and glands of the rat uterus.

Fig. 6. Localization of \( \text{CaT1} \) expression in the rat uterus. In situ hybridization was performed, as described in MATERIALS AND METHODS, to detect \( \text{CaT1} \) mRNA in the uteri of rats at proestrus (Pro), diestrus (Di), and estrus (Es) (A) and in the uteri of \( P_4 \)-treated rats (B). Arrows indicate positive cells.
CaBP-9k and CaBP-28k, which are crucial factors for early embryo implantation, are expressed on the same uterine layer (14, 15). According to these data, uterine CaT1 may have two possible functions: CaT1 may take part in embryo implantation at the window of receptivity and/or act as a calcium regulator to secrete substances into the uterine lumen such as the exocrine organs. CaT1 expression levels in the human placenta were shown to be much higher in the kidney and small intestine (6). This higher CaT1 placental expression is consistent with transplacental calcium ion transport and is critical for normal fetal growth and development (6).

In summary, uterine CaT1 expression increases at diestrus. P4, a predominant hormone at diestrus, is a major mediator in the regulation of CaT1 gene expression in the uterus of immature and OVX rat models. In a time-dependent experiment, a significant increase in CaT1 mRNA is observed at 12 h after the final P4 treatment. In addition, P4 induces uterine CaT1 transcription via a PR-dependent pathway, as shown by the use of a PR-specific antagonist. Using in situ hybridization, we have observed CaT1 transcript in the endothelial layer and glandular epithelium of the rat uterus. Together, these results suggest that CaT1 may be regulated by P4 at diestrus via a PR-dependent pathway, to take part in uterine function.

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