行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※
※Suppression of Na\(^+\)/K\(^+\) ATPase activity is associated with the decrease※
※
※ of contraction frequency in late gestation uteri
※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：x 個別型計畫 □整合型計畫
計畫編號：NSC-89－2320－B－006－068
執行期間： 88年08月01日至89年07月31日

計畫主持人：蔡美玲

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

執行單位：成功大學

中華民國89年10月30日
行政院國家科學委員會專題研究計畫成果報告
國科會專題研究計畫成果報告撰寫格式說明
Preparation of NSC Project Reports
計畫編號：NSC 89-2320-B-006-068
執行期限：88年8月1日至89年7月31日
主持人：蔡美玲
計畫參與人員：張明佑、李佳玲、黃冰玉

一、中文摘要

懷孕末期子宮節律收縮頻率下降，已知抑制鈉鉀幫浦活性降低節律收縮頻率，因此本研究之目的乃探討懷孕末期子宮節律收縮頻率下降與鈉鉀幫浦活性降低之關係。利用 ATPase assay，西方式黑色素染色，肌肉收縮計器測量鈉鉀幫浦酵素活性，蛋白質含量，及肌肉收縮力。早期懷孕子宮較晚期懷孕子宮表現高之肌肉收縮頻率。肌肉收縮頻率低。早期懷孕子宮較晚期懷孕子宮表現高之鈉鉀幫浦蛋白質含量及鈉鉀幫浦酵素活性。若以無鉀溶液短期抑制鈉鉀幫浦酵素活性會引發節律收縮，但鈉鉀幫浦酵素活性會引發節律收縮。若以低鉀溶液活化鈉鉀幫浦酵素活性會引發節律收縮。早期懷孕子宮對無鉀溶液或低鉀溶液之反應遠較較早期懷孕子宮來得明顯。經雌性素處理，抑制節律收縮，鈉鉀幫浦蛋白質含量及鈉鉀幫浦酵素活性。已知早期懷孕時血清中雌性素遠較晚期懷孕時高，因此本研究推論晚期懷孕時雌性素的增加，抑制鈉鉀幫浦酵素活性及鈉鉀幫浦蛋白質含量導致肌肉收縮頻率下降。

關鍵詞：子宮、懷孕、鈉鉀幫浦、節律收縮、老鼠

Abstract

Contraction frequency of spontaneous oscillations decreases in late gestation uteri. Na⁺/K⁺-ATPase, consisted of α and β subunits, is involved in agonist-induced oscillatory contraction. The purpose of this study was to examine whether the decrease in Na⁺/K⁺-ATPase activity contributed to the reduction of spontaneous oscillations in late gestation uteri. Contraction apparatus, ATPase assay, and Western blot analysis measured contraction force, enzyme activity of Na⁺/K⁺-ATPase, and protein abundance of Na⁺/K⁺-ATPase α subunits, respectively. Early gestation uteri exhibited higher contraction frequency than late gestation uteri. Gestation day 7 (G7) uteri (as early gestation) exhibited higher contraction frequency than gestation day 18 (G18) uteri (as late gestation). In conjunction, the former contained higher Na⁺/K⁺-ATPase activity and protein abundance of Na⁺/K⁺-ATPase α 1 subunit when compared to the latter. Inhibition of Na⁺/K⁺-ATPase by potassium-free solution and ouabain increased contraction frequency immediately. More than 40 min exposure to potassium-free solution suppressed oscillations. Activation of Na⁺/K⁺-ATPase by lower concentrations of potassium induced oscillatory contractions. When compared with G18 uteri, G7 uteri were more susceptible to Na⁺/K⁺-ATPase modulators. Taken together, the decrease in the expression of Na⁺/K⁺-ATPase α 1 subunit, which leads to the decrease of Na⁺/K⁺-ATPase activity. The reduction of Na⁺/K⁺-ATPase activity may be associated with the occurrence of uterine quiescence in late gestation.

Keywords: uterus, pregnancy, Na⁺/K⁺-ATPase, rat, contraction, oscillations.

二、緣由與目的
After implantation, the pregnant uterus undergoes a series of modifications to accommodate the developing fetus. With the progression of gestation, the contraction frequency of spontaneous oscillations is decreased in conjunction with the increase in the size of conceptus. Finally, the pregnant uterus becomes quiescent during the second half of pregnancy (Fuchs, 1969). Right before term, the uterus develops synchronized contraction to expel the conceptus. Several mechanisms account for the development of synchronized contraction before term including the increase of oxytocin receptors (Fuchs et al., 1985) and the formation of gap junctions (Garfield et al., 1977). However, the possible mechanism responsible for the decrease of spontaneous uterine oscillations during the late gestation is still not clear.

Structurally Na+/K+-ATPase is composed of a catalytic α and a glycosylated β subunits (Lingrel et al., 1990; Sweadner, 1989). The former contains the binding sites for Na+, K+, Pi and ouabain (a specific inhibitor for Na+/K+-ATPase (Eakle, 1992) and the latter may help to assemble and transport the α subunit to the plasma membrane (Ueno et al., 1997). Activation of Na+/K+-ATPase triggers the mobilization of two K+ into and three Na+ out of the cells (Ewart et al., 1995). Current reports demonstrate that Na+/K+-ATPase is involved in relaxation phase of agonist-induced oscillatory contractions. In agonist-induced oscillations, phasic components appear on the top of tonic contractions. High concentrations of ouabain (1 mM) suppressed oscillations (Kong et al., 1990; Gustafsson et al., 1994). All the pharmacological data suggest that the reduction of Na+/K+-ATPase activity suppresses agonist-induced oscillatory contraction.

Na+/K+-ATPase is known to exist in rat uterus (Turi et al., 1992; Herrera et al., 1987). Its activity and mRNA expression of Na+/K+-ATPase α subunit is changed with gestation period. In vivo treatment with 17β-estradiol for 2 days decreases Na+/K+-ATPase activity in conjunction with the decrease of spontaneous uterine oscillations (Tsai et al., 2000, submitted). When compared to early gestation, the plasma level of 17β-estradiol is significantly elevated in late gestation. In conjunction, the oscillatory activity in late-gestation uteri decreases. Therefore, the purpose of this study was to examine whether the decrease in Na+/K+-ATPase activity contributes to the reduction in spontaneous contractions of late-gestation uteri. Our first objectives were to measure the influence of pregnancy on the contraction frequency of spontaneous oscillations, the protein abundance of Na+/K+-ATPase α subunit, and the enzyme activity in pregnant uteri. Secondly, the contraction responses to the modulators of Na+/K+-ATPase activity in pregnant uteri were examined: ouabain and K+-free solution to inhibit and lower concentrations of KCl (less than 4.6 mM) to activate Na+/K+-ATPase activity.

三、材料與方法

Animal Care and Models
Female Sprague-Dawley rats weighing 200-300 g housed in the colony at The Animal Center of National Cheng Kung University Medical College at 24±1 °C under a 14-h light (0500-1900) schedule were used in these experiments. All animals studied were performed under protocols and procedures approved by local Animal Care and Use Committee and in accord with NIH standards established by the Guidelines for the Care and Use of Experimental Animals and by the American Veterinary Medical Association.

Estrogen-treated rats: Virgin rats with the age between 60 and 90 days were ovariectomized following anesthetization with ether. Two weeks later, the ovariectomized rats were divided into three groups. Each group was daily subjected to 17β-estradiol (5 μg/ml/kg) or solvent (as control) subcutaneously. The stock solution of 17β-estradiol was dissolved in sesame oil for daily use. After a 4-day treatment, rat uteri were removed for experiments.

Pregnant Rats: Virgin female rats at proestrus stage (determined by virginal smear) were housed with a male rat. The day of
observed virginal plug was designed as day 0 (G0). Because the full term for SD rats is about 21-22 days, rats on gestation day 7 (G7, as early gestation) and 18 (G18, as late gestation) were used. All rats were subjected to light ether anesthesia before sacrifice. Antimesometrical portion of each uterine horn was further separated into implantation and non-implantation regions (close to oviducts) and then cut into longitudinal uterine strips (1 mm wide by 15 mm long). Some uterine strips were further divided into two parts: one for measuring contraction force and the other for preparing tissue homogenates.

**Preparation of tissue homogenates**

The method for preparing tissue homogenates was modified from the method of Turi et al. (1985). Uterine horns were kept in homogenizing solution of the following composition: 150 mM-sucrose, 30-mM histidine, 1 mM EGTA, 1% deoxycholate, and 0.1 M PMSF. After adipose tissues were trimmed, the minced uteri were ground with Tissue Tearor (Cole-Parmer, IL) and then homogenized with a Pestle/Tube homogenizer (Cole-Parmer, IL). After centrifugation at 10,000 g for 20 min, the pellet was discarded. The supernatant was further centrifuged at 100,000 g for 60 min. After the centrifugation was removed, the pellet was resuspended in 50 mM Tris-HCl (pH 7.2). The tissue homogenate was further divided into two parts: one for measuring the enzyme activity and the other for Western blot analysis. Rat brain homogenate prepared in the same manner were used as a positive control.

**Immunoblot analysis of Na⁺/K⁺-ATPase α1 subunit**

The method for immunoblot analysis has been described previously (Tsai et al., 2000). In brief, 70 μg protein/lane of uterine tissue homogenate or 5 μg protein/lane of brain tissue homogenate was subjected to polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate and then transferred to polyvinylidifluoride membranes by electroblotting. After incubating in phosphate-buffered saline (136.9 mM-NaCl, 2.7 mM-KCl, 10 mM- Na₂HPO₄, 1.8 mM-KH₂PO₄) containing 5% non-fat milk (Carnation) and 0.5% (vol/vol) Tween 20 for 1.5 hr at room temperature to block nonspecific binding, the blots were probed with antibodies against the α1- subunit of Na⁺/K⁺-ATPase. The blots were treated with secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) labeled with peroxidase (dilution 1:10000) for 50 min. After the treatment with chemiluminescence reagent according to the manufacturer’s protocol (ECL, Amersham), the blots were exposed to X-ray film to visualize protein bands. The molecular weight of each protein band was determined by comparison with prestained protein marker (Amersham) run simultaneously with the experimental lanes. The protein abundance of β-actin was also assessed as an internal control for protein quantification. The expression of Na⁺/K⁺-ATPase α1 subunit from rat brain was served as a positive control. A background control was obtained when the primary antibodies were not added. Mouse anti-rabbit Na⁺/K⁺-ATPase α1 monoclonal antibody (1:1000) (Upstate Biotechnology, NY) was used.

**Quantification**

Multiple exposures of autoradiograms were made to ensure that signals were within the linear range of the film. A computerized image analyzer (Quantity One, PD1 Inc., NY) quantified the intensity of autoradiographic bands on immunoblots. After scanning, the OD reading of Na⁺/K⁺-ATPase α1 subunit was normalized by the OD from its own β-actin and expressed as a ratio. The ratio from G7 was defined as 100%.

**Na⁺/K⁺ ATPase Assays**

The Na⁺/K⁺ ATPase activity was determined according to the method of Tsai et al. In brief, tissue homogenates were transferred to an incubation medium of the following composition: 10 mM-MgCl₂, 3.3 mM-EDTA, 100 mM Tris (pH 7.8) and then divided into two tubes. One-half of the homogenate was mixed with the preheated assay mixture with 10 mM ouabain and the other half was mixed with the same mixture without ouabain. The assay mixture
contained 1.132 M NaCl, 0.2 M KCl, 0.05 M Na3 and ATP. Fifteen minutes later, adding 200 μl of 30% tricholoacetic acid stopped the reaction. The ouabain-insensitive and sensitive phosphate liberation was measured by spectrophotometer at a wavelength of 660nm. The difference in the liberation amount between ouabain-insensitive and sensitive phosphate can be attributed to the presence of Na+/K+-ATPase enzyme activity in the ouabain-sensitive phosphate. Protein was measured by the method of Lowry et al. (1954), using bovine albumin as the standard.

**Measurement of Uterine Contractions**

A longitudinal uterine strip 1 mm wide by 15 mm long was taken from the anteriomesometrial side of a horn and placed in physiological salt solution (PSS). The composition of the PSS was as follows: 116 mM NaCl, 4.6 mM KCl, 1.16 mM NaH2PO4·H2O, 1.16 mM MgSO4·7H2O, 21.9 mM NaHCO3, 1.8 mM CaCl2·2H2O, 11.6 mM dextrose, and 0.03 mM CaNa2EDTA.

The uterine strips were mounted in an organ bath containing PSS for isometric force measurement as described previously (Tsai et al., 1996). The PSS was maintained at 37°C and was aerated with a mixture of 95% O2 and 5% CO2. All preparations were allowed to equilibrate for at least 40 min under a constant passive force of 1.0 g. This level of passive force was determined to be optimal for maximum force development to 60 mM potassium chloride (KCl). After a 40-min equilibration period, all strips were challenged with 60 mM KCl for determining viability and maximum contractile force. Those strips that did not respond to KCl were discarded. A cycle was operationally defined as the generation of force of at least two-thirds of the KCl (60 mM)-induced contraction that initiated and returned to baseline.

**Data Analysis and Statistical Evaluation**

All data are expressed as means ± SEM (standard errors of the means). The data were analyzed by one-way analyses of variance (ANOVA). If the mean values were found statistically different, Least Standard Difference test in the Means model of SYSTAT was used for multiple comparisons. In all cases, a p value less than 0.05 was considered statistically significant.

四・結果

**Effect of Pregnancy on Spontaneous Contraction and the expression of Na+/K+-ATPase α 1 subunit in Rat Uteri**

To characterize the effect of pregnancy on the contraction frequency of spontaneous oscillations, pregnant rats on gestation day 0 (G0), 7 (G7), 10 (G10), 14 (G14), 18 (G18), 21 (G21) were used. Corresponding to the changes in contraction frequency, the protein abundance of Na+/K+-ATPase α subunit in G0, G7, G10, G14, G18, G21 uteri was measured. All pregnant uteri exhibit spontaneous oscillations. The contraction frequency of G0 was about 7 cycles/10 min. Compared to G0 uteri, the contraction frequency was significantly lower in G14 and G18 uteri but higher in G21 uteri (fig. 1). In conjunction, Na+/K+-ATPase α subunit existed in all pregnant uteri. The protein abundance of Na+/K+-ATPase α subunit in uteri of G14 and G18 was much lower than that in those of other stages (fig.2). Our data show the association between contraction frequency of spontaneous contraction and the expression of Na+/K+-ATPase α subunits. Because the protein abundance of Na+/K+-ATPase α subunits in G7 was lower than that in G18 uteri, we further measure the enzyme activity of Na+/K+-ATPase. As fig.3 indicated, the enzyme activity in G7 was about 6.5 μmole/mg/hr, which was significantly higher than that in G18 uteri. G7 uteri containing higher Na+/K+-ATPase would exhibit greater oscillatory responses to Na+/K+-ATPase modulators than G18 uteri. Our data suggest that the decrease of Na+/K+-ATPase is associated with the suppression of spontaneous oscillations in late gestation uteri.

**Effect of Na+/K+-ATPase modulators on Contraction Frequency of Pregnant Uteri**

If Na+/K+-ATPase is involved in the
maintenance of spontaneous oscillations, we speculated that G7 uteri containing higher Na\(^+/\)K\(^+/\)-ATPase activity were more responsive to Na\(^+/\)K\(^+/\)-ATPase modulators than G18 uteri containing less Na\(^+/\)K\(^+/\)-ATPase activity.

In response to increasing concentrations of ouabain from 10 \(\mu\)M to 0.3 mM, the contraction frequency of G7 was increased in a concentration-dependent manner but G18 was not (fig. 4). Ouabain at 0.3 mM induced much greater frequency in G7 uteri than that in G18 uteri. Right after exposure to K\(^+/\)-solution, both G7 and G18 uteri developed uterine contractions. The latency of responding to K\(^+/\)-free solution was much shorter in G7 uteri than that in G18 uterus. The duration of oscillatory contractions in G7 was much longer than that in G18 uteri. After long term inhibition by K\(^+/\)-free solution, contraction frequency of oscillatory contraction was suppressed (fig. 5). As our data indicated, G18 uteri were less responsive to the inhibitors of Na\(^+/\)K\(^+/\)-ATPase than G18 uteri.

After exposure to K\(^+/\)-free solution for more than 1-h, the addition of lower concentrations of potassium from 0.3 to 1.2 mM induced oscillatory contractions in G7 uteri but did not in G18 (fig. 6). At 4.6 mM (within a physiological range) KCl significantly increased contraction frequency in G7 uteri than that in G18 uteri. The data support our speculation that the G7 uteri were more responsive to Na\(^+/\)K\(^+/\)-ATPase modulators than G18 uteri.

To exclude the possibility that the differential response to lower concentrations of KCl was due to the change in membrane potential, KCl, a depolarization agent, was used. In response to increasing concentrations of potassium from 3 to 12 mM, contraction frequency of G7 and G18 uteri was increased in concentration-dependent manner. However, the responsiveness of G7 uteri to KCl was not different from that of G18 (Fig. 7). The data excluded the possibility that the differential membrane potential contributes to the decrease of spontaneous oscillations in late gestation uteri.

**Correlation between Na\(^+/\)K\(^+/\)-ATPase activity and ouabain-induced oscillations in estrogen-treated uteri**

After the in vivo treatment with 17\(\beta\)-estradiol for 4 days, the contraction frequency of ouabain-induced contractions was suppressed (fig. 7A). In conjunction, the enzyme activity of Na\(^+/\)K\(^+/\)-ATPase was less in estrogen-treated uteri than that in ovariecetomized uteri (Fig. 7B). The data suggest that the decrease in Na\(^+/\)K\(^+/\)-ATPase activity was associated with the decrease in spontaneous oscillations in uteri.

五、討論

The contraction pattern of spontaneous oscillation in pregnant uteri changed with the progression of gestation. As we know, uteri at term develop synchronized oscillations: regular but high-frequency contractions. When compared with the uteri at term, the early gestation uteri develop irregular but higher frequency contractions. The late gestation uteri develop regular but lower-frequency contractions (Fuchs, 1969; Downing et al., 1981). Our data in Fig. 1 agreed with their reports that G14 and G18 exhibit lower and G21 exhibits higher contraction frequency. Downing et al (1978) demonstrated that in vivo effect of 17\(\beta\)-estradiol at the doses of 0.25 to 5 \(\mu\)g reduces the frequency of spontaneous uterine contraction but increases the contraction response to oxytocin. Therefore, the increased 17\(\beta\)-estradiol in late gestation has been thought to influence contraction frequency of spontaneous oscillations in late gestation uteri. Our data agree with previous data that early gestation uteri exposing to lower level of estrogens elicit greater contraction frequency of spontaneous contraction than late gestation uteri exposing to higher level of estrogens.

Even though the elevation of estrogen with the progression of pregnancy is involved in the decreased frequency of spontaneous contraction in rats, the mechanisms responsible for the long-term effects of estrogens on the decrease of spontaneous oscillations in pregnant uteri are not clear.
However, estrogens are known to increase the expression of potassium channels in uteri, which then increases membrane potential. If the reduction of spontaneous oscillations in late gestation uteri was due to the increase in abundance of potassium channels, the membrane potential in G7 uteri would be lower than that in G18 uteri. Kuriyama et al. (1965) demonstrated that the membrane potential in G7 uteri is about 64 mV, which is similar to that in G18. Our data (Fig. 4) indicate that the contraction response of G7 uteri to the depolarization agent, KCl, was no difference from that of G18 uteri. As these findings suggest, it is unlikely that the decrease of contraction frequency in late gestation uteri is due to the increase of potassium channels.

Estrogens are known to inhibit Na\(^{+}/K\(^{+}\)-ATPase activity in intestine and uteri (Tsai et al., 2000). The inhibition of Na\(^{+}/K\(^{+}\)-ATPase attenuates the relaxation phase of agonist-induced oscillations (Kong et al., 1990; Ausina et al. 1996; Gustafsson et al., 1994). It is plausible to hypothesize that the increase in late gestation reduced Na\(^{+}/K\(^{+}\)-ATPase activity, which then attenuated the relaxation phase of spontaneous oscillations and inhibits contraction frequency of spontaneous oscillations. When compared to late gestation uteri, the early gestation uteri exposing to lower plasma level of estrogens. In conjunction, G18 contains lower enzyme activity and protein abundance of Na\(^{+}/K\(^{+}\)-ATPase than G7 uteri. The duration of each contraction-relaxation cycle was longer in the G18 uterus than that in the G7 uterus. The contraction frequency of spontaneous oscillations was lower in G18 uteri than that in G7 uteri. The contraction responses to Na\(^{+}/K\(^{+}\)-ATPase modulators were less in G18 uteri than those in G7 uteri. Likewise, estrogen-treated uteri exhibits lower Na\(^{+}/K\(^{+}\)-ATPase activity. In conjunctions, the 4-day treatment with 17β-estradiol decreased the contraction frequency of ouabain-induced oscillations. The data (Fig. 7, 8) support that the decrease of Na\(^{+}/K\(^{+}\)-ATPase in late gestation uteri may, in part, contribute to the decreased frequency of spontaneous oscillations in late gestation.

If the expression of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit in pregnant uteri was modulated by the elevated estrogens, we may speculate the expression of the expression of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit was suppressed in G18 uteri because 17β-estradiol was maintained at the higher level. When compared to the protein abundance of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit in G7 uteri, G18 uteri elicits lower contraction frequency and the expression of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit. Our finding indeed supports that the elevation of 17β-estradiol in late gestation may result in the reduction of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit and activity, which, in turn, lower contraction frequency.

If the elevation of 17β-estradiol in late gestation is the major factor to reduce the expression of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit and activity, we should measure a further decrease in the protein abundance of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit in G21 uteri. However, the amount of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit in G21 uteri was close to that in G7 uteri. When considering the hormonal profile in late pregnancy, 17β-estradiol is gradually elevated from mid gestation and maintains at plateau in late gestation. In contrast, progesterone is elevated right after implantation and maintains at plateau throughout gestation. Right before parturition (G21 in rats), the plasma level of progesterone is dramatically decreased to about the level at estrous stage. Current reports demonstrate that progesterone inhibit Na\(^{+}/K\(^{+}\)-ATPase activity in various tissues (Longerich et al., 1988; Mujais et al., 1993; Weiland et al., 1991). Therefore, we speculate that the increase of Na\(^{+}/K\(^{+}\)-ATPase α1 subunit in G21 uteri may be due to the decrease of progesterone. In addition, our preliminary data also showed Na\(^{+}/K\(^{+}\)-ATPase activity was decreased by progesterone in the presence of estrogens. However, the influence of progesterone on Na\(^{+}/K\(^{+}\)-ATPase subunits in uteri needs further investigation.

In conclusion, this is the first report to demonstrate the presence of Na\(^{+}/K\(^{+}\)-ATPase α1 in pregnant uteri may be involved
in the development of spontaneous oscillatory contraction in pregnant uteri.
With the progression of gestation period, the elevation of estrogen may influence the decrease in contraction frequency in late gestation uteri. Right before parturition, the induction of $\text{Na}^+/\text{K}^+$-ATPase $\alpha_1$ and oscillatory contractions requires some factors other than estrogens.

六、参考文献


