Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

The effects of apelin on myometrium contractions in pregnant rats

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Received October 23, 2017; **Accepted** August 16, 2018; **Published** August 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.11.13

Abstract: Apelin, which is a new hormone, is secreted especially in the brain by hypothalamus as well as by many other organs like the stomach, fat tissue, and the heart. For apelin, whose possible effects on many bodily functions like the endocrine system, cardiovascular system and metabolic activities are still under investigation, the reproductive system is also an important target area. The purpose of the present study was to investigate the effects of plasma apelin levels in rats that were in diestrus, pregnancy and lactation periods, and to examine its possible effects on myometrium contractions of pregnant rats and **non-pregnant** rats that were in diestrus period. The plasma apelin concentrations in female adult Wistar rats were determined with the ELISA method in the diestrus period, and on the 12th, 18th, and 21st days of the pregnancy, and on the 2nd and 10th days of lactation (n=7 for each group). In addition, the effect of apelin at 0.01, 0.1, 1 and 10 µM doses on isometric contractions in the rat uterus on the 21st day of pregnancy and in diestrus period was tested by using isolated organ bath. This protocol was repeated under conditions that were pre-treated with protein kinase C inhibitor in calcium-free medium, and the possible effect of apelin on contractions and the mechanisms that might mediate this effect were investigated. When plasma apelin levels were compared with the rats in diestrus period, the apelin concentrations in the 21-day pregnancy group were high at a significant level ($p<0.05$); and low at a significant level in the 2-day lactation group ($p<0.05$). In myometrium contraction trials, it was observed that apelin induced the contractions. Apelin increased the frequency of the myometrium contractions at a significant level when applied at 1 µM and 10 µM concentrations ($p<0.05$ and $p<0.001$). Only after the apelin application at 10 µM concentration did the amplitude of the contractions increase at a significant level ($p<0.01$). In the myometrium of the rats that were on the 21st day of pregnancy, the frequency of the contractions was statistically significant at 0.1 µM, 1 µM and 10 µM doses ($p<0.01$). In addition, the amplitude of the contractions increased at a statistically significant level at 1 µM and 10 µM dose application ($p<0.05$ and $p<0.01$, respectively). The apelin application induced the contractions in calcium-free medium. When apelin was applied after the pre-application with protein kinase C inhibitor, no contractions were observed. The present study showed that apelin levels were increased at the end of pregnancy in rats, and the hormone induced the uterus contractions. This effect does not occur with protein kinase C inhibitor and in calcium-free medium, which shows that protein kinase C pathway might play a role in these mechanism. These findings show that apelin might be an endogenous peptide that plays a role on uterine contractions at birth in rats.

Key words: Apelin; Contraction; Myometrium; Pregnancy; Tissue Bath.

Introduction

Apelin was first defined in 1998 by Tatemoto et al. (1). It shows its effects through APJ receptor (2). Apelin is secreted by many organs like stomach, fat tissue, kidney, lung, placenta, mammary glands and heart (3-5) and especially by the hypothalamus in brain (6). Apelin has important physiological effects on endo-crine and metabolic functions. In this study, the plasma apelin levels in rats in diestrus, pregnancy and lactation periods, and its effects on myometrium contractions in pregnant and non-pregnant rats were investigated.

Apelin mRNA expression has been reported in studies conducted on the effects of apelin in reproductive system in ovary follicles, and in different developmental stages of the granulosa cells, and the APJ expression has been reported to increase at a significant level in granulosa cells of estrogen inactive dominant follicles when compared with the other follicles. Apelin and APJ exist in intragluteal artery smooth muscles, and show their effect through angiogenic factors like vascular endothelial growth factor and fibroblast growth factor; and they also show their angiogenic effects in corpus luteum (7). With this effect, apelin causes proliferation of the capillary and the increase of the nutrient and pioneering substances, which altogether lead to development of dominant follicle (7). APJ has been shown to exist in human placenta in the 1st and 3rd trimester (8). Although apelin expression in normal placenta shows a decrease in the placental villus, cytotrophoblast and sincytotrophoblasts from the 1st trimester till the 3rd trimester of the pregnancy, APJ expression level increases in the cytoplasm of the cytotrophoblasts and the endothelial cells of the placenta. The high apelin concentration level being high at a clear level in umbilical plasma sample supports the role of apelin in the intrauterine development. The sharp decrease in the apelin level in the circulation on postnatal 1st day results from the removal of the placenta (9).

The fact that apelin has different plasma concentrations throughout pregnancy period (10), and being expressed at high levels in supraoptic and paraventricular

nucleus, which are oxytocin synthesis areas in rat hypothalamus (6), and the existence of APJ expression in rat myometrium (11) supports that this hormone might be influential in pregnancy and birth processes. But, the source of plasma apelin is not clear. Especially cardiac endothelial cells and adipose tissue may be main source of apelin in blood. Increasing adipose tissue at the end of pregnancy and placenta can secrete more apelin. It's obvious that apelin locally synthesized in uterus or coming from blood plasma act in myometrium by binding its' receptors.

Many factors play roles in pregnancy and birth processes in mammals. These factors associated with physiological mechanisms in agreement with each other makes it possible for a healthy birth function (12). On the other hand, the real mechanism of the birth or the events that start the birth are still not well known (12). All of the hormones including progesterone, estrogen, cortisol, relaxin, oxytocin, corticotrophin-secreting hormone (CRH), prostaglandin and catecholamine affect the start and sustaining of the birth and make it possible for uterus to be emptied in the end (12). Many points that are still not known about the efficiency of these mechanisms await being clarified. The aim of the present study was to examine possible effects of apelin, which is a new hormone that was acquired by the world of science in recent years, on some physiological events related with pregnancy and birth process. In addition, it was considered that questioning whether the intracellular pathway might play an intermediary role in possible effects of apelin will provide additional evidence for some of the effects of apelin that are already known in the intracellular communication although the effect mechanisms of it has not been fully elucidated. Determination of the plasma apelin concentrations in rats during pregnancy and lactation will also contribute to the understanding of possible effects of this hormone in these processes. With this study, important steps will be taken on designing future studies on the interaction among-metabolism-obesity-pregnancy-birth. Possible effects of apelin on myometrium contractions in rats that are in diestrus period and that are pregnant were examined in the present study. The fact that there are few studies conducted on the effect of apelin on uterus contractions in rats increases the scientific value of the present study. Determination of the plasma apelin concentrations in rats that are in pregnancy and lactation periods will also provide new insight to better understand possible effects of this new hormone on these processes.

Materials and Methods

The approval of the local ethical board was received in the present study, and Wistar adult intact female rats weighing 200-250 gr were used. The rats were obtained from Fırat University, Experimental Research Center. The estrus cycles of the rats were observed between 08.00-09.00 in the morning on a daily basis with vaginal smear. The rats that were in pro-estrus cycle were placed in the cages as 1 male 1 female in each cage. The following morning, the animals that had sperms in the vaginal swab were accepted as being on the day 0 of pregnancy. The animals whose pregnancies were on the same date were kept in same cages and followed until the experiment day. The experiments were performed in 2 different protocols. In the first protocol, 1 ml blood samples were collected from the rats that were in the diestrus period, and on 12th, 18th and 21st days of pregnancy under anesthesia, and from the rats that were on the 2nd and 10th days of lactation (except for the group that was pregnant for 21 days) to determine the plasma apelin concentration. In the 2nd protocol, the uteruses of the rats that were pregnant for 21 days and the rats that were in the diestrus period were used for contraction experiments. 12x2x1 mm myometrium stripes were placed in isolated organ baths in which there was Krebs-Henseleit solution (the contents: mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.25, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) and 95% O_2, 5% CO_2. The baths were constantly gassed. The contractions were recorded with physiological power convertor (FDT05, Commat Ltd., Ankara, Turkey) and with MP150WS Windows (Biopac Systems Inc, CA, the USA). Apelin was purchased from Sigma (St. Louis, MO, the USA), intracellular inhibitor and chelerythrine chloride were purchased from Tocris Bioscience. Each stock solution was dissolved in required amounts before applying in the organ bath.

Plasma apelin-13 levels were determined by using the Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cat. No. S-1416, BACHEM, the UK) in the light of the instructions of the manufacturer company. The absorbance of the samples was measured at VERSA (Designed by molecular Devices in California, the USA) brand micro plate read at 450 nm wavelength. The results were determined as ng/mL. The measurement range of the kit was 0-100 ng/mL.

Statistical analysis

All values were determined as Mean ± Standard Deviation (Mean ± SD). Statistical analyses were made by using the SPSS 21.0 Program. Statistical evaluations were made by using One-Way Variance analysis. The $p<0.05$ value was taken to be significant in all analyses.

Results

First protocol group results

The plasma apelin levels in all groups were determined as Mean ± SD from the blood samples by using the ELISA method (n=7 for each group). The apelin levels in the group that was pregnant for 21 days were higher at a statistically significant level when compared with the group that was in the diestrus period and the group that was pregnant for 12 days ($p<0.05$, Figure 1). There was no significant difference in the group that was pregnant for 18 days. The apelin concentration in the 2-day lactation group was lower at a statistically significant level when compared with the values measured on the 21st day of pregnancy ($p<0.05$, Figure 1). No significant differences were detected in the rats that were on the 10th day of lactation.

The second protocol group results

The myometrium sections that were taken from the rats in diestrus period and the rats that were on the 21st day of pregnancy were placed in the organ bath chamber that contained Krebs Solution. The apelin doses in 0.01 μM, 0.1 μM, 1 μM and 10 μM concentrations were

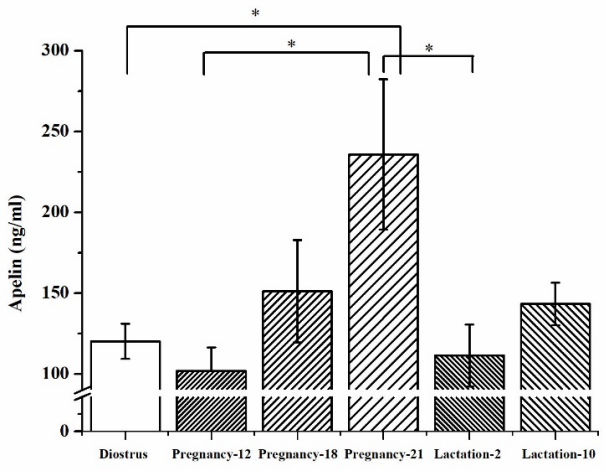

Figure 1. The plasma apelin levels in the rats on the Diestrus Period; on the 12th, 18th and 21st Day of pregnancy, and on the 2nd and 10th days of lactation, $p<0.05$. One-Way Variance Analysis (n=7 for each group).

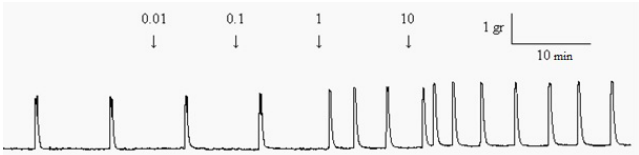

Figure 2. The effect of apelin at 0.01 μM, 0.1 μM, 1 μM and 10 μM concentrations on the spontaneous contractions of the myometrium of the rats that were in diestrus period (n=7 for each group).

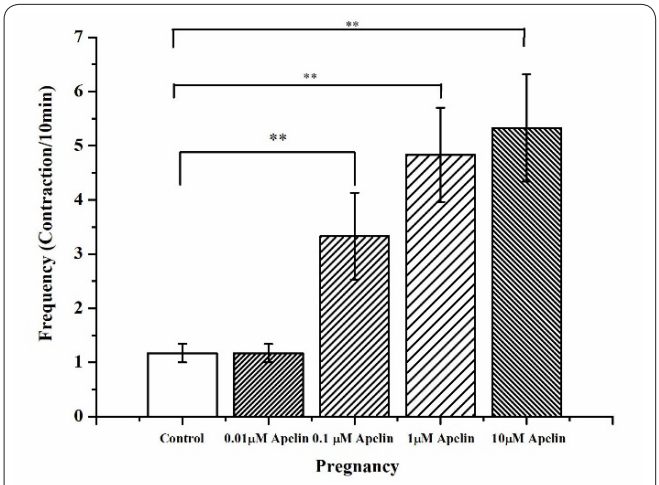

Figure 3. The effects of apelin on the frequencies of uterus contractions in rats that were pregnant for 21 days, $p<0.01$**One-Way Variance Analysis (n=7 for each group).

added to the bath chamber in a cumulative manner with 10-minute periods (n=7 for each group). It was observed that the apelin applied in 1 μM and 10 μM doses to the myometrium of the rats that were in the diestrus period increased the frequency of the contractions at a significant level ($p<0.05$ and $p<0.001$, respectively; Figure 2). Only after the apelin application at 10 μM concentration, it was observed that the amplitude of the contractions increased at a significant level ($p<0.01$). Apelin was statistically significant when given at 0.1 μM, 1 μM and 10 μM doses in myometrium of the rats that were on the 21st day of pregnancy ($p<0.01$) (Figure 3).

Apelin at 0.1 μM, 1 μM and 10 μM doses led to a clear increase in the contraction frequency ($p<0.01$; Figure 3). No differences were detected in the other group

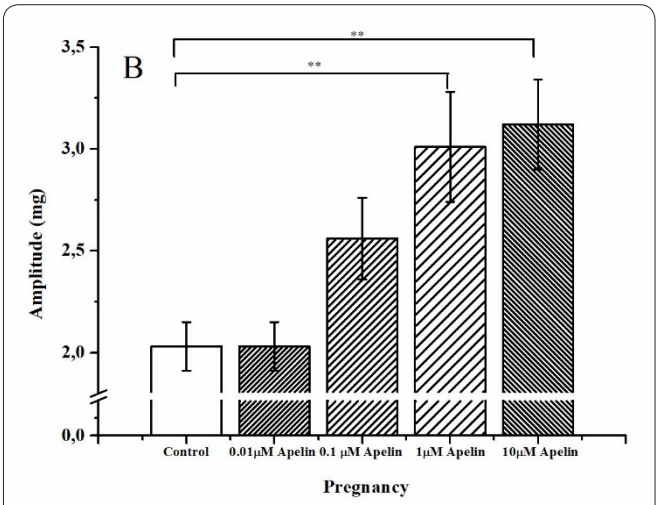

Figure 4. The effects of apelin on the amplitude of uterus contractions in the rats that are pregnant for 21 days; $p<0.05$ and $p<0.01$ *One-Way Variance Analysis (n=7 for each group).

at a statistically significant level.

Apelin at 1 μM ($p<0.05$) and 10 μM ($p<0.01$) concentrations caused a clear increase in the amplitude of contractions (Figure 4). In other groups, no statistically significant differences were detected.

After the second protocol experimental stage, the myometrium stripes were placed in the bath chamber with the same method. The spontaneous contractions became visible. Right at this stage, Krebs solution without Ca^{+2} was added to the organ bath, and it was observed that the spontaneous contractions stopped immediately. After 10-minute control recording, apelin was applied to the bath chamber at 10 μM concentration, and the changes that occurred were recorded (n=6). Only one contraction occurred in all applications as a result of 10 μM apelin application (Figure 5).

Chelerythrine chloride, which is a PKC inhibitor was applied at 10mM dose after the control recording in which the contractions did not occur in the experimental design in Krebs solution without Ca^{+2}. Five minutes after c*helerythrine chloride*, 10 μM apelin was added. However, after *chelerythrine chloride* pre-application, no contractions occurred after apelin was applied (unlike the group to which *chelerythrine chloride* was not

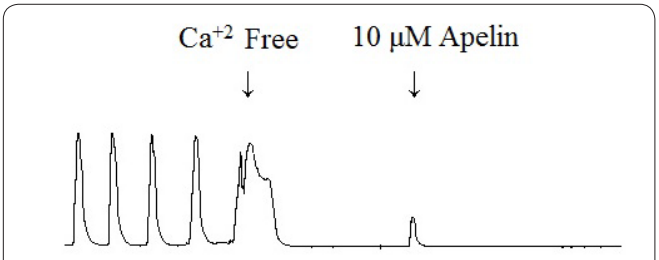

Figure 5. The original trace that shows its effect at 10 μM apelin concentrations in Krebs Solution without Ca^{+2}.

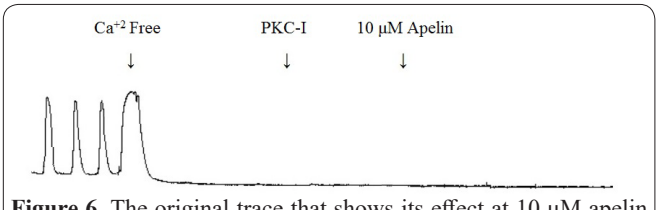

Figure 6. The original trace that shows its effect at 10 μM apelin concentrations in Krebs Solution without Ca^{+2}.

applied, Figure 6).

Discussion

Pregnancy and birth processes consist of a chain of events in which many complex physiological mechanisms play active roles in mammals. Especially the basic triggering mechanisms that make birth process start have not been explained fully yet. However, the oxytocin secretion from the hypothalamo-hypophyseal system, the pressure caused by fetus or fetuses in the uterus increasing on the uterus wall and thus stimulating spontaneous myometrium contractions, the upregulation of the oxytocin receptors in the myometrium in the last stage of pregnancy, and several mechanisms like the estrogen/progesterone ratio being changed in favor of the estrogen may be influential in this process. Understanding pathophysiological mechanisms related with some obstetric diseases is of crucial importance for treatment-focused modalities. This will only be possible by understanding the basic factors about the end-stage of pregnancy and birth.

In this study, it was shown that apelin has a clear stimulator effect on *in vitro* uterus contractions in rats that are at the end stage of pregnancy. Apelin performs its effect most probably by inducing Ca^{+2} release from intra-cells depots; because in a medium that lacks Ca^{+2}, apelin induces the myometrium contractions. This effect of apelin appears most probably through the intracellular pathway. Apelin did not induce contractions in a medium which was pre-treated with *chelerytine chloride*, which plays an intermediary role in the stimulator effect of apelin on myometrium contractions, might be the intracellular activation. This pathway is one of the basic factors for the cellular effect mechanisms of apelin, because it has been shown in previous studies that apelin activates intracellular pathway in the cell in cardiomyocytes (13).

Calcium ions play great roles in the regulation of contractions in myometrial cells, which is the case in skeletal muscle. However, since sarcoplasmic reticulum (SR) is not developed well in smooth muscles, the calcium that is needed for contractions comes mostly from the extracellular fluid. Right before the phasic contractions in the myometrium, the intracellular calcium level increases with the Ca^{+2} entry from the extracellular medium (14, 15, 16). It has been shown in previous studies that if there is no Ca^{+2} in the medium in which the myometrium sections exist, spontaneous contractions stop suddenly and spontaneous activity disappears (16).

In the present study, the plasma apelin levels of the rats that were in the diestrus period, and in different periods of pregnancy and lactation were also examined. It was determined that the plasma apelin level increased at the end-stage of pregnancy at a significant level when compared with the diestrus and 12^{th} day of pregnancy. Although Van Mieghem et al. showed that maternal apelin concentration decreases at a rate of 50% in rats in which previously these rates were normal (17); in our findings, apelin hormone increased towards the end of pregnancy. Similar to our findings, Telejko et al. and Kourtis at al. (18, 19) showed that apelin plasma level and the placenta apelin mRNA expression increased towards the end of pregnancy. The increase in apelin towards birth makes us consider that it might contribute to the induction of the myometrium contractions.

In studies conducted on the reproductive system, it has been reported that FSH inhibits APJ expression in *in vitro* cultured granulose cells. It has been shown that LH stimulates the expression of apelin and APJ mRNA expression in theca cells; and progesterone stimulates the APJ expression in granulose cells (19). In addition to these, plasma apelin level increasing throughout pregnancy period has made us consider that apelin may have a role in the intrauterine development. In the plasma sample, the apelin level being high at an obvious level, and the intrauterine growth retardation when the apelin level is low, and the pre-eclampsia development in such a situation support the role of apelin in intrauterine development (20). In addition, although apelin has been detected in mRNA tissues, the highest expression has been detected in the mammary gland of pregnant rats (9). It has also been determined that apelin is expressed at a high level in the mammary glands of the rats that are in lactation period (9). The apelin and apelin mRNA levels increase parallel to the mammary glands throughout pregnancy, and reach peak levels at the end of pregnancy and at birth (9). After birth, the apelin levels are also high in mammary glands, and decrease to the basal level within 21 days (9). Apelin has also been detected in human breast milk (9). Apelin secretion in great amounts has been shown in bovine colostrum (21). In the light of these data and the data determined in our present study, apelin seems to contribute to the birth in mammals.

In vitro and *in vivo*, apelin causes the phosphorylation of Myosin Light Chain Kinase (MLC) in smooth muscle cells in a dose-dependent manner (22). It increases the amplitude of the Ca^{+2} activity in rat myositis, and reduces the calcium content in the SR during the electrical stimulation. intracellular has an important contribution in this effect of apelin. While the reduction in the calcium content in SR occurs with a mechanism that is dependent on intracellular, the increase in the Ca^{+2} activity is independent from PKC (23). This might be a possible underlying mechanism contributing to birth by stimulating the uterus contractions like oxytocin. In the trials, spontaneous contractions occurred in the myometrium with the application of apelin at increasing doses in media with and without calcium in rats that were in the diestrus period and that were in the end-stage of pregnancy. In the rats that were in the end-stage of pregnancy, while the frequency and amplitude of the contractions were significantly high when compared with the rats in the diestrus group, a single dose of apelin caused contraction in the Ca^{+2}-free medium. This stimulating effect that occurs in spontaneous contractions makes us consider that apelin hormone causes that the intracellular calcium increases in myometrial cells. Based on these findings, it might be claimed that apelin induces contractions by ensuring that calcium is released from intracellular depots in isolated myometrium of female rats that are not pregnant. Some previous studies have reported that apelin shows some physiological effects by increasing intracellular calcium concentrations in some cells. Choe et al. showed that apelin increased the intracellular calcium level in human brain

neuron cell cultures (24). Medhurst et al. reported that apelin peptides increased the intracellular calcium in RBL-2H3 cells derived from rat basophile (3).

While apelin stimulates the contractions in the uterus, it shows a hypotensive effect on blood vessels, which constitute another smooth muscle type, by causing vasodilatation. The hypotensive effect of apelin occurs through NO/L-arginine system (25). Apelin stimulates the endothelial nitric oxide synthase phosphorylation in endothelial cells. NO activates guanyl cyclase, cGMP level increases, and relaxation occurs in the vascular smooth muscles (25). Apelin causes different effects with different mechanisms in two different smooth muscles. All these data make us consider that apelin might have several different receptor types.

In conclusion, although apelin does not show any effects on contractions in rat myometrium at low doses, it shows an increasing effect on the amplitude and frequency of the spontaneous contractions in a dose-dependent manner. Apelin shows its stimulating effects on rat myometrium even when extracellular Ca^{+2} is absent, which means that it shows its effect by inducing calcium release from intracellular depots. When PKC inhibitor is applied, apelin does not have any effects, which means that apelin shows its possible effect through intracellular.

Funding
This work was supported by FUBAP–1963.

Conflicts of interest
There is no conflicts of interest.

Acknowledgements
Each author has participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and conduct of the experiment and for data interpretation.

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