Summary of Ph.D. Thesis

NEURAL REGULATION OF UTERINE CONTRACTILITY IN THE RAT: THE ROLE OF ADRENERGIC AND SENSORY NERVES

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Szeged

2004

INTRODUCTION

Control of uterine smooth muscle contractility is of vital importance during pregnancy and parturition. The musculature of the uterus is characterized by a relative quiescence throughout gestation until the onset of labor. At this time powerful contractions and the dilation of the uterine cervix occur which result in the expulsion of the fetus and the placenta. In a significant number of pregnancies, however, premature labor and uterine dystocia commence as a result of pathologies of still poorly understood pathogenesis. Since premature labor is still considered to be the leading cause of neonatal morbidity and mortality, a better understanding of the factors which may affect and regulate uterine contractility would further promote the development of novel strategies in the treatment of this condition.

The rat genital organs are densely innervated by autonomic nerves, which reach the uterus via the hypogastric and the pelvic plexa. These nerves have different innervation territories: the uterine horns are mostly innervated by the hypogastric nerve, while the cervix and the vaginal canal are mostly supplied by the pelvic nerve. Hypogastric and pelvic nerves carry mainly sympathetic and parasympathetic postganglionic axons, respectively. The sympathetic fibers contain noradrenaline as a principal transmitter. The varicose unmyelinated noradrenergic fibers innervate predominantly the blood vessels via axons running in the adventitia, but many of them course freely into the myometrial and the endometrial layers. They can be demonstrated either by catecholamine fluorescence histochemistry using paraformaldehyde gas and/or glyoxylic acid or by the immunohistochemical localization of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis.

At the end of pregnancy, the numbers of both myometrial and perivascular adrenergic nerve fibers are markedly reduced in the rat, and the guinea pig, and also in humans. Although this neuroplastic change in the innervation pattern of the pregnant uterus is known for a long time, the exact mechanism and time course of this phenomenon are not yet fully understood. At advanced pregnancy the noradrenaline content is significantly reduced in various portions of the rat uterus, both in the vicinity of and distant from the fetuses. The time course of the structural denervation process and its consequence on the uterine contractility remains to be elucidated.

Uterine sensory nerves may be stimulated by intense mechanical and chemical stimuli which produce pain. The afferent nerves of the uterus are involved in the mediation of nociceptive impulses as well as in reflex actions involving the uterine cervix at labor. These unmyelinated C- and Aδ-type sensory fibers of the uterus and the cervix also travel in the hypogastric and pelvic nerves. In many

organs and tissues the physiological role of sensory nerves is not limited to their "afferent function", i.e. the transmission of noxious information from the periphery to the central nervous system. They also possess an “efferent” or “local regulatory” function through the release of a variety of neuropeptides involving substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A (NKA), and galanin (GAL). In most visceral organs, the stimulation of sensory nerves – via the release of these neuropeptides - elicits significant alterations in smooth muscle contractile activity. Similarly as sympathetic nerves, certain types of sensory nerve fibers in the rat uterus may undergo degeneration in the course of pregnancy. However, there is some controversy as to the nature and extent of these changes.

A large population of sensory neurons express the capsaicin/VR1/TRPV1 receptor and is exquisitely sensitive to the stimulatory and neurotoxic action of capsaicin and related vanilloids. Capsaicin-sensitive afferent nerves have been demonstrated by immunohistochemistry in the uterus of several species including the rat. A significant proportion of these nerves contain SP, CGRP, NKA, and GAL. Although sensory neuropeptides which may be released from capsaicin-sensitive afferent nerves affect uterine smooth muscle function, the possible participation of these particular afferent nerves in the modulation of uterine contractility has not been dealt with in detail, yet.

AIMS OF THE STUDY

1. The aim of the present study was to investigate the time course of pregnancy-induced changes in the contractile responses of isolated uterine rings and in the sympathetic innervation pattern, using electric field stimulation and histofluorescence techniques, respectively. The functional consequences of adrenergic denervation on myometrial contractility were studied on days 5, 10, 15, 18, and 22 of pregnancy. The uterine adrenergic nerve function was characterized by measuring uterine contractile responses under in vitro conditions using electric field stimulation.

2. Experiments were also initiated in an attempt to reveal a possible role of capsaicin-sensitive peptidergic sensory nerves in the modulation of uterine contractility in non-pregnant and term pregnant rats. Selective nerve-induced contractions were elicited and the effect of chemodenervation by capsaicin on the contractile responses of uterine preparations was examined. Immunhistochemistry was applied to localize CGRP in intact and capsaicin treated uteri in order to

demonstrate the possible depletion of CGRP from capsaicin-sensitve afferent nerves innervating uterine blood vessels and the myometrium.

MATERIALS AND METHODS

Animals and mating

Sexually mature female Sprague-Dawley rats (body mass: 180-240 g, 60-80 days old) were used in the experiments. Nonpregnant rats were cyclic virgin rats in diestrus phase, which was detected by taking vaginal smears from the animals in the early morning hours. Mating with males was carried out in the early morning hours. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The day of conception was considered to be the first day of pregnancy.

Investigation of adrenergic innervation

Animals

The uterine adrenergic innervation was studied in intact nonpregnant and pregnant rats. Tissue samples from pregnant rats were obtained on days 5, 10, 15, 18, and 22 (term) of pregnancy.

Uterus preparation and electric field stimulation parameters

The rats were killed by cervical dislocation at 10 a.m. and the uteri were removed and trimmed of fat; in uteri obtained from pregnant rats the feto-placental units were removed. The uterus was immediately placed in an organ bath (de Jongh's solution containing, in mM: NaCl 137, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 12, Na_2HPO_4 4 and glucose 6; pH 7.4*),* perfused with 95% oxygen and 5% carbon dioxide. The temperature of the organ bath was maintained at 37°C. Rings 1 cm in length were cut from the middle part of each horn, including implantation sites in the case of pregnant uteri, and mounted vertically between two platinum electrodes in the organ bath. After mounting, the uterine rings were equilibrated for 90 min before the start of the stimulation. The initial tension was set at 1.5 g. After the incubation period, selective nerve stimulation was elicited by a digital, programmable stimulator (ST-02, Experimetria Ltd., London, U.K.). The square pulse duration was set at 0.6 ms and the supramaximal voltage at 40 V.

Rings were stimulated by 1-min trains of square pulses with the following frequencies, in increasing order: 1; 2.5; 5; 10; 20; 30; 50; 70 Hz. Each 1-min stimulation was followed by a 4-min

recovery period, whereafter a higher frequency was applied. The tension of the myometrial rings was measured with a strain gauge transducer (SG-02, Experimetria Ltd., London, U.K.) and recorded and analyzed by ISOSYS Data Acquisition System (Experimetria Ltd., London, U.K.). In a series of experiments uterine preparations were stimulated as described above, but in the presence of 1 μM phentolamine.

Histofluorescence examination of uterine adrenergic innervation

Nonpregnant animals and pregnant rats on days 5, 10, 15, 18 and 22 of pregnancy were killed and bled. The uteri were removed, placed on cryostat chucks and frozen. Longitudinal sections 15 μm in thickness were cut and mounted on glass slides. Specimens were immersed for about 3s in a solution of 0.1 M glyoxylic acid containing in mM: KH_2PO_4 235 and sucrose 200, pH 7.4. Air-dried sections were either covered with liquid paraffin and placed in an oven at 95 °C for 2.5 min or dried over phosphorus pentoxide overnight and then reacted with paraformaldehyde vapour at 80 °C for 60 min. The preparations were examined under a Leitz fluorescence microscope equipped with appropriate filters.

Studies on the role of capsaicin-sensitive nerve fibers in uterine function

Animals

The uterine capsaicin-sensitive nerve fibers were studied in intact nonpregnant, 22-day pregnant, and in capsaicin-treated nonpregnant rats. Capsaicin was administered to adult nonpregnant female rats in three subcutaneous injections at doses of 10, 20 and 100 mg/kg on days 1, 2 and 4, respectively, under ether anesthesia. The experiments were performed four days after the last dose.

Uterus preparation and electric field stimulation parameters

Isolated uterine ring preparations were prepared as described above, then stimulated by 30-s trains of square pulses (of 0.6 ms duration, at a supramaximal voltage of 40 V) with the following frequencies, in increasing order: 1; 2.5; 5; 10; 20; 30; 50 and 70 Hz. Each 30s stimulation period was followed by a 2-min recovery period, whereafter a higher frequency was applied. The contractions elicited by the first series of stimulations were regarded as the control responses. The tissue samples were washed thoroughly and then incubated for 20 min with capsaicin at final concentrations of 0.1-10 μM. Thereafter, the uterine rings were washed with the physiological solution and stimulated again

with the same series of frequencies (1-70 Hz). The tension of the myometrial rings was measured with a strain gauge transducer and recorded and analyzed by ISOSYS Data Acquisition System. The changes of the amplitude of the contractions after the exposure to capsaicin were expressed as the percentage of the corresponding control responses.

In some experiments on intact nonpregnant rat uteri, the second series of stimulations were performed in the presence of the $CGRP_1$ receptor subtype antagonist, hCGRP(8-37).

Immunohistochemistry

Uterine rings were placed in an organ bath containing de Jongh's solution perfused with 95% oxygen and 5% carbon dioxide, at 37^{o}C. Thirty min later, capsaicin was added at final concentrations of 1-10 μM. Solvent-treated specimens served as controls. After an additional period of 20 min this solution was replaced with fresh physiological solution. Ten min later, tissue specimens were placed in Zamboni's fixative for 2 h at 4^{o}C. The samples were washed in phosphate buffer (0.1 M, pH 7.4) with 10% sucrose added and processed for the demonstration of CGRP-immunoreactivity (-IR) using an indirect immunofluorescence technique. Briefly, cryostat sections 15 μm in thickness were cut and incubated with a rabbit polyclonal CGRP antibody (1:500) overnight. After repeated washing with phosphate buffered saline the sections were incubated with a secondary antibody (goat anti-rabbit IgG conjugated to Cy3) for 1 h at room temperature. After washing in buffer, tissue sections were covered with Cytifluor and examined under the fluorescence microscope using appropriate filters.

Statistical analysis

All experiments were carried out on at least six animals and values are given as mean ± S.E.M. Bartlett tests revealed the homogenity of variances. One-way ANOVA with Newman-Keuls test was used to compare the amplitudes of given contractile responses at each frequency independently. The recorded data were statistically analyzed with the Prism 2.01 (Graph Pad Software Inc., San Diego, CA) computer programme.

RESULTS

Investigation of adrenergic innervation

The findings demonstrate a progressive, frequency-related reduction of nerve-mediated uterine contractions beginning in midterm pregnancy, in parallel with a gradual loss of adrenergic nerve fibers. At all frequencies, the amplitudes of contractions were highest in nonpregnant uteri. Stimulation at 1-2.5 Hz evoked contractions in 10-day pregnant uteri but failed to to cause contractions on day 5 and from day 15 onward. In phentolamine-treated uterine preparations and in the uteri of term pregnant rats contractions could not be evoked by stimulation at frequencies lower than 20 Hz. Fluorescence histochemistry of uterine adrenergic nerves revealed a rich perivascular and myometrial innervation in nonpregnant and in pregnant rats through day 10. Degeneration and loss of adrenergic nerve fibers was apparent by day 15, and fluorescent myometrial and perivascular nerves were practically absent by day 22. The reduced responsiveness of uterine smooth muscle to electric field stimulation in early pregnancy (day 5) appeared to be unrelated to alterations in uterine innervation but may be related to changes associated with implantation.

Studies on the role of capsaicin-sensitive nerve fibers in uterine function

In nonpregnant rats, the amplitudes of uterine contractions dose-dependently increased after a 20-min incubation period with capsaicin (0.1-1 μM), which was possibly due to partial neuropeptide release. However, no change in the amplitude of uterine contractions was observed when uterine specimens were incubated with capsaicin at 10 μM, which was supposedly the consequence of a destruction of sensory nerve endings by capsaicin. Prior systemic treatment of the rats with capsaicin abolished the contraction-increasing effect of in vitro capsaicin administration on the amplitude of neurally evoked contractions, suggesting that the effect of capsaicin on uterine contractility was of neural origin.

When the uterine contractions were evoked by electric field stimulation in the presence of the CGRP-antagonist hCGRP(8-37), the amplitudes of contractions elicited at 1-30 Hz were significantly higher than in the control, resulting in an augmentation of amplitudes similar to the effects of the in vitro capsaicin treatment. The greatest enhancement of the amplitude was observed when the uterine rings were stimulated with 1 Hz in the presence of 10 μM hCGRP(8-37). If the uterine samples were stimulated at higher frequencies, the effect of hCGRP(8-37) on the amplitude of contractions was significantly smaller than that of 1 μM capsaicin.

In agreement with previous findings, CGRP-immunohistochemistry revealed a dense innervation of uterine blood vessels and smooth muscle in control (solvent-treated) specimens. Exposure to capsaicin at concentrations of 0.1-1 μM produced a marked but incomplete depletion of CGRP both from perivascular and myometrial nerve fibers. In contrast, capsaicin administered at a concentration of 10 μM produced an almost complete disappearance of all CGRP-IR nerves from the tissue. Exposure to capsaicin produced similar effects in the term pregnant rat uterus, although the density of CGRP-IR nerve fibres was markedly reduced as compared to nonpregnant control rats.

In the term pregnant rat uterus low-frequency stimulation (< 20 Hz) was unable to elicit contractions, which was probably due to the pregnancy-induced denervatiion process. However, stimulation at 20-70 Hz revealed that certain populations of capsaicin-sensitive sensory nerves did not degenerate with pregnancy. The effect of 1 μM capsaicin on the amplitude of contractions was quite similar to that in the nonpregnant uteri: a significant increase of the amplitude was observed at each frequency (20-70 Hz), as compared with the solvent-treated control. Similarly as in the nonpregnant uteri, incubation with 10 μM capsaicin did not alter the amplitude of the contraction responses.

DISCUSSION

Previous morphological investigations revealed the innervation patterns of the nonpregnant, the term pregnant and the postpartum rat uterus and disclosed a marked decrease in the number of adrenergic nerves in the pregnant uterus. However, the time course of these degenerative changes in the rat uterus has not been dealt with in detail. Here we investigated both the functional and the structural changes in the adrenergic innervation in the rat uterus on selected days of pregnancy. The myometrial function was described by the presence of nerve-induced contractions: the lack of a contractile response to stimulation at certain frequencies was considered to be a failure of adrenergic nerve function. The functional alterations could also be interpreted in terms of morphological changes. Our observations indicated that the diminution of the contractility correlated with the progressive loss of uterine adrenergic nerve fibers. Fluorescence histochemistry and electric field stimulation showed that, similar to nonpregnant uteri, myometrial adrenergic nerve fibers are morphologically intact and functional up to day 10 of pregnancy. The denervation process commenced in mid-pregnancy and progressed towards term. The results suggest that contractions mediated by adrenergic nerves do not play a significant role in controlling myometrial motor activity from midpregnancy until term.

Studies of the time course of the degeneration process of adrenergic nerves revealed a parallelism between the functional and morphological changes of the rat myometrium, except for the 5th day of pregnancy. At this time, low-frequency nerve stimulation failed to elicit contractile responses, although the histological picture of the 5-day pregnant myometrium showed an apparently intact adrenergic innervation. Although the uterine adrenergic innervation in the 5-day pregnant myometrium seems to be similar to that in the nonpregnant myometrium, adrenergic nerves may differ in their function, e.g. in excitability or in excitation-contraction coupling.

It is suggested that there is a critical balance between the synaptic and nonsynaptic adrenergic control of the uterine function in rats during pregnancy. The adrenergic system has a dual function in controlling uterine contractility: protection of the developing fetus and initiation of labor contractions. The physiological significance of adrenergic nerve degeneration is still enigmatic; the possible factors initiating this process might involve the fetuses themselves (as physical stretch).

Characterization of uterine contractile responses under in vitro conditions using electric field stimulation revealed that adrenergic contractions may be elicited at lower frequencies of 1-10 Hz. It is generally accepted that stimulation at higher frequencies activates mainly peptide containing afferent nerves. We suggest, therefore, that contractile responses elicited at higher frequencies result mainly from the activation of sensory nerves in the rat uterus and, like in most visceral organs, can be attributed to the release of neuropeptide(s) from sensory nerves.

Our observations showed that capsaicin-sensitive primary afferents in the nonpregnant rat uterus can be stimulated at a wide range of frequencies. In the uteri of intact nonpregnant rats, but not in the uteri of nonpregnant rats systemically treated with capsaicin, the in vitro capsaicin treatment caused a dose-dependent increase of the amplitudes of uterine contractions. These findings clearly demonstrate that the contractility-increasing effect of capsaicin is fully dependent on an intact sensory nerve function, and it can be regarded as an indirect action on the myometrium. Our findings suggest that the increased amplitude of responses after in vitro capsaicin treatment is a consequence, at least partially, of the capsaicin-induced release and depletion of CGRP from the sensory nerve endings. Probably, the changes in the tissue levels of CGRP play a significant role in the mechanism of the capsaicin-induced augmentation of uterine contractile responses.

The pregnancy-induced alteration of sensory nerve function in the uterus is a sparsely investigated area, although the consequences of enhanced contractility closely before term may involve preterm labor. The remaining contraction responses in term pregnant rat uteri also proved to be higher

after incubation with 0.1 or 1 μM capsaicin, similarly as in the nonpregnant rat uteri, which supports the notion that certain populations of sensory nerves in the rat uterus do not degenerate with pregnancy.

The mechanism of contractility enhancement due to neuropeptide depletion from stimulated afferent nerves may be of particular interest, as regards the mechanism of preterm delivery. Hence, low pH of inflamed tissue may stimulate sensory nerve endings and result in a consequent depletion/release of sensory peptides. Considering that in approximately 25% of all preterm births a genital infection is the most probable trigger factor, the further study of the involvement of sensory nerves in uterine pathology may offer a promising approach to a better understanding of the role of sensory nerves in uterine function under physiological and pathological conditions. Finally, pharmacological targeting of uterine sensory nerve function may offer a novel way of modulation of uterine motor control.

ACKNOWLEDGEMENTS

I owe my thanks to my supervisor, **Professor George Falkay**, the head of the Department of Pharmacodynamics and Biopharmacy, who has been supporting my work with caring attention.
I wish to express my gratitude to **Professor László Kovács**, the head of the Reproductive Health Ph.D. Programme, for the possibility to take part in the Ph.D. studies.
I would like to offer my sincere thanks to **Professor Gábor Jancsó** for his ongoing interest, advice and encouragement, and for critically reviewing the manuscript.
I am grateful to my colleagues, **Róbert Gáspár Ph.D.** for his generous help and his never failing attention, and **Péter Sántha Ph.D.** for the inspiring discussions. Thanks to **Ferenc Domoki Ph.D.** who has arisen my interest in life sciences.
Thanks are due to my colleagues in the Department of Pharmacodynamics and Biopharmacy, and in the Department of Physiology for the favourable athmosphere.

I am deeply grateful to my family for their patience and loving care.

This work was supported by the Hungarian Research Grants OTKA T033126, OTKA T032507, and by the Richter Gedeon Centennial Foundation.

ANNEX

Full papers and abstracts related to the Ph.D. thesis

I. **Klukovits A.**, Mike L., Gáspár R., Falkay Gy.: Az adrenerg denerváció hatása terhes patkány uterusz kontraktilitására *in vitro*. Acta Pharmaceutica Hungarica 2001; 71:181-186.

II. **A. Klukovits**, R. Gáspár, P. Sántha, G. Jancsó, G. Falkay: Functional and histochemical characterization of a uterine adrenergic denervation process in pregnant rats. Biology of Reproduction 2002; 67:1013-1017.

III. **A. Klukovits**, R. Gáspár, P. Sántha, G. Jancsó, G. Falkay: Role of capsaicin-sensitive nerve fibers in uterine contractility in the rat. Biology of Reproduction 2004; 70:132-138.

IV. **Klukovits A.**, Gáspár R., Jancsó G., Falkay Gy: A terhességi adrenerg denerváció funkcionális és morfológiai jellemzése patkány uterusában. A Magyar Élettani Társaság LXVI. Vándorgyűlése, Szeged, 2001. június 6-8.

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VIII. **A. Klukovits**, R. Gáspár, P. Sántha, G. Jancsó, G. Falkay: The modulation of uterine contractile activity by capsaicin-sensitive afferent nerves. 4th Congress of the Federation of European Physiological Societies, 28 June – 3 July, 2003; Nice, France.

IX. **A. Klukovits**, E. Ducza, I. Földesi, G. Falkay: Tocolysis with β_2-adrenoceptor agonists induce oxytocin receptor up-regulation in the late-pregnant rat uterus in vivo and in vitro. Acta Pharmaceutica Turcica 2002; 44 (Suppl).