Investigation of pregnancy induced adrenergic denervation in the rat

PhD Thesis

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LIST OF SCIENTIFIC PUBLICATIONS RELATED TO THE SUBJECTS OF THE THESIS

1. Zupkó I, Csonka D, Falkay G: A rat model for functional characterization of pregnancy-induced denervation and postpartum reinnervation in the myometrium and cervix: a superfusion study. *Reproduction* **130:** 743-9 (2005) IF_{2005}: 3.136

2. Csonka D, Zupkó I, Minorics R, Márki A, Csík G, Falkay G: The effects of α-methyldopa on myometrial noradrenaline release and myometrial contractility in rat. *Acta Obstet Gynecol Scand* **86:** 986-94 (2007) IF_{2007}: 1.274

3. Csonka D, Kormányos Z, Csík G, Zupkó I, Falkay G: Szuperfúziós technika alkalmazása a myometriális adrenerg transzmisszió vizsgálatára. *Magy Nőorv L* **71:** 121-6 (2008)

ADDITIONAL PUBLICATIONS

1. Mihályi A, Gáspár R, Csonka D, Falkay G: Synergism between beta 2-adrenoceptor agonists and subtype-selective alpha 1A-adrenoceptor antagonists in the tocolytic effect on pregnant rat uterus in vitro. *Clin Exp Pharmacol Physiol* **30:** 164-7 (2003) IF_{2003}: 1.744

2. Falkay G, Spiegl G, Csonka D, Zupkó I: Effects of streptozotocin-induced diabetes on the uterine adrenergic nerve function in pregnant rats. A superfusion study. *Neurochem Int* **51:** 306-10 (2007) IF_{2007}: 2.975

3. Spiegl G, Zupkó I, Minorics R, Csík G, Csonka D, Falkay G: Effects of experimentally induced diabetes mellitus on pharmacologically and electrically elicited myometrial contractility. *Clin Exp Pharmacol Physiol* **36:** 884-91 (2009) IF_{2008}: 2.196

List of abbreviations

ACE – Angiotensin Converting Enzyme
ANOVA – Analysis of Variance
APGAR –Appearance, Pulse, Grimace, Activity, Respiratory Effort
ATII – Angiotensin Type 2
B_{max} – Number of receptors present in the sample
CGRP – Calcitonin Gene-Related Peptide
CRH – Corticotropin Releasing Hormone
CRHBP – Corticotropin Releasing Hormone Binding Protein
dpm – disintegrations per minute
EC_{50} – Median Effective Concentration
EFS – Electric Field Stimulation
E_{max} – Maximal Obtainable Effect
HELLP – Hemolysis, Elevated Liver Enzymes, Low Platelets
K_d – Dissociation constant
MAO – Mono Amine Oxidase
MHC – Major Histocompatibility Complex
mRNA – Messenger Ribonucleic Acid
NGF – Nerve Growth Factor
SEM – Standard Error of Mean
VIP – Vasoactive Intestinal Peptide

Contents

Introduction

Regulation of myometrial contractility

Pregnancy is one of the most mysterious phenomenon of our life. There are many unresolved questions covering all stages of pregnancy. This is the reason why pregnancy is researched from multiple points of view. Plenty of biochemical, physiological, pathophysiological and pharmocological methods are used to answer the open questions of physiological as well as pathological conditions affecting pregnancy e.g. preterm birth (Simhan and Caritis, 2007, Yeast and Lu, 2007, Goldenberg *et al.*, 2008, Iams *et al.*, 2008), hypertensive disorders in pregnancy like preeclampsia (Baumwell and Karumanchi, 2007, Bonney, 2007), or other forms of gestational hypertension (Frishman *et al.*, 2005, Podymow and August, 2007) and gestational diabetes (Hawkins and Casey, 2007).

During pregnancy, the uterus is maintained in a state of functional quiescence through the action of various putative inhibitors, like progesterone, prostacyclin, relaxin, nitric oxide, CGRP, VIP. Before term, uterus undergoes activation and stimulation. Activation occurs in response to uterotropins including oestrogen. Once activated, uterus can be stimulated to contract by uterotonins such as oxytocin and prostaglandin E_2 and $F_{2\alpha}$. Involution of the uterus after delivery is mediated primarily by oxytocin (Norwitz *et al.*, 1999).

The development of placenta is a common feature of reproduction in most mammals, but variations on the theme of parturition among placental mammals are considerable.

In most viviparous animals the fetus is in control of the timing of labor via the fetal pituitary adrenal axis (Thorburn *et al.*, 1991). However the placenta of humans lacks the glucocorticoid-inducible enzyme 17α-hydroxylase-17,20-lyase, which is critical to this pathway, and thus this mechanism does not apply to humans.

In humans by contrast the timing of birth is associated with the development of the placenta – in particular with the expression of corticotropin releasing hormone (CRH) by the placenta.

Several studies have shown an association between levels of maternal plasma CRH, which is of placental origin, and the timing of birth. Maternal plasma CRH levels increase exponentially as pregnancy advances, peaking at the time of delivery. Production of CRH by the placenta is restricted to primates. Humans and great apes also produce a circulating binding protein for CRH (CRHBP). At the end of pregnancy, CRHBP levels fall, thereby increasing the bioavailability of CRH. Placental CRH production is modified by cortisol,

estrogen, progesteron and nitric oxide which are inhibitory and by a range of neuropeptides which are stimulatory (Smith, 2007).

Another important event in labor is the expression of a group of protein termed „contraction-associated proteins". These proteins act within the uterus which is in a relaxed state for most of pregnancy, to initiate the powerful rhythmic contractions that force the fetus through a softening cervix at term. There are three types of contraction-associated proteins: those that enhance the interactions between the actin and myosin proteins that cause muscle contraction, those that increase the excitability of individual myometrial cells, and those that promote the intercellular connectivity that permits the development of synchronous contractions.

During pregnancy, the growth of the uterus under the action of estrogens gives the fetus space for its own growth, but uterine growth ceases toward the end of pregnancy, and the consequent increasing tension of the uterine wall signals the onset of parturition. In most smooth muscle organs, stretching leads to contraction. The switch of the growth-accomodating behavior of the uterus during most of the pregnancy to the stretch induced by the cessation of uterine growth at labor appears to be regulated by progesterone. It is likely that progesteron withdrawal increases the attachment of myocytes to the intercellular matrix, through integrins, and this process promotes activation of mitogen-associated protein kinase and increases contractility. Progesterone plays a critical role in the development of the endometrium by allowing implantation and subsequently, the maintenance of myometrial relaxation. In many mammals, a drop in circulating progesterone levels precipitates parturition, in humans the level of circulating progesterone does not fall with the onset of labor, however the progesterone antagonist RU486 can initiate parturition at any time during pregnancy. A search for mechanisms that could account for a functional withdrawal of progesterone has identified several forms of the progesterone receptors. With the onset of labor the proportions of progesterone receptors change in a way that could constitute a mechanism of progesterone withdrawal.

A better understanding of the pathway to normal birth should provide the basis for identifying points along the pathway at which a pathological process may precipitate preterm birth. The road to an understanding of human birth is challenging. It is important to determine which pregnancies carry a risk of preterm birth and to intervene with appropriate measures (Smith, 2007).

Spontaneous preterm birth is a physiologically heterogeneous syndrome. The cascade of events that culminate in spontaneous preterm birth has several possible underlying pathways. Four of these pathways are supported by a considerable amount of clinical and experimental evidence: excessive myometrial and fetal membrane overdistention, decidual hemorrhage, precocious fetal endocrine activation, and intrauterine infection or inflammation. These pathways may be initiated weeks to months before clinically apparent preterm labor. The processes leading to preterm parturition may originate from one or more of these pathways. Our understanding of the nature of the molecular cross-talk among these pathways is in its infancy. The etiologic heterogeneity of preterm birth adds complexity to therapeutic approaches. Our knowledge regarding the identification and treatment of women at high risk for preterm labor has critical gaps and therefore this area is a target of future research (Smith, 2007).

Another unresolved question of pregnancy is how the immune tolerance of the mother is developing. There are several proposed mechanisms to explain how the fetus can evade immune rejection by the mother. One of them is that the maternal immune system might not be capable of responding to fetal antigens due to mechanisms that induce anergy or tolerance in responding maternal cells. Several studies investigated this mechanism and despite their efforts the results of the studies conflict and the mechanisms causing changes to the maternal T cell compartment during pregnancy remain unclear.

Second is that an anatomical barrier might form between mother and fetus preventing access of maternal immune cells to fetal antigens. This could be the trophoblast which separates mother and fetus. The barrier formed by the trophoblast is complex with multiple different cell types. Trophoblast cells resist immune attack, and the differentiated cells of trophoblast are also frequently renewed so any immune injury that does occur might be quickly repaired. It was hypothesized that this fetal-maternal barrier prevents the maternal immune system from contacting fetal cells baring alloantigen. However some fetal cells do enter the maternal circulation and can persist for years and even decades in the mother.

Third, fetal cells might suppress the expression of alloantigens. Fetal cells might evade detection and thus destruction by the maternal immune system by down-regulating the expression of fetal alloantigens. This was based on the fact that trophoblast cells in contact with maternal circulation do not express either MHC class I or II molecules. While the lack of expression of classical MHC molecules might help to explain the survival of the fetus, it

cannot completely explain the ability of the fetus to evade destruction by the maternal immune system. When the expression of MHC class I molecules were induced on trophoblast, the maternal immune system did not reject the fetus. Thus, some mechanisms other than immunological ignorance also protects the fetus from maternal immune system.

Fourth, recently proposed, that the fetus generates site-specific immune suppression. This means that the effector functions of maternal immune cells would be blocked at the maternal-fetal interface protecting the fetus while allowing the cells to mount immune responses to microorganisms and other foreign cells. Elucidating the mechanisms important for successful pregnancy would benefit not only reproductive immunology but other fields as well (Koch and Platt, 2007).

Hypertensive complications of pregnancy

As a group hypertensive disorders represent the most significant complication of pregnancy, affecting approximately 10% of all pregnancies and contributing greatly to maternal and perinatal morbidity and mortality throughout the world. Fifteen percent of maternal deaths in the US are solely the result of hypertensive disease, which can lead to iatrogenic preterm delivery for maternal indications as well as adverse fetal outcomes. Hypertension during pregnancy carries with it the increased risk of abruption placentae, disseminated intravascular coagulation, cerebral hemorrhage, hepatic failure and acute renal failure (Frishman *et al.*, 2005).

In 2000, four categories of hypertension in pregnancy have been defined: chronic hypertension, gestational hypertension, preeclampsia and preeclampsia superimposed on chronic hypertension. Chronic hypertension is defined as a blood pressure measurement of 140/90 mmHg or more on two occasions before 20 weeks of gestation or persisting beyond 12 weeks postpartum. Treatment of mild to moderate chronic hypertension neither benefits the fetus nor prevents preeclampsia. Excessively lowering blood pressure may result in decreased placental perfusion and adverse perinatal outcomes. When a patient's blood pressure is persistently greater than 150 to 180 / 100 to 110 mmHg pharmacologic treatment is needed to prevent maternal end-organ damage. Methyldopa, labetalol and nifedipine are oral agents commonly used to treat chronic hypertension in pregnancy.

Gestational hypertension has replaced the term pregnancy-induced to describe women who develop hypertension without proteinuria after 20 weeks of gestation. Gestational

hypertension is a provisional diagnosis that includes women eventually diagnosed with preeclampsia or chronic hypertension, as well as women retrospectively diagnosed with transient hypertension of pregnancy. Fifty percent of women diagnosed with gestational hypertension between 24 and 35 weeks develop preeclampsia.

Preeclampsia is a multiorgan disease process by unknown etiology characterized by the development of hypertension and proteinuria after 20 weeks of gestation. Some of the proposed theories of pathogenesis are abnormal placental implantation, angiogenic factors, cardiovascular maladaptation and vasoconstriction, genetic predisposition, platelet activation, immunologic intolerance between fetus and mother, vascular endothelial damage or dysfunction. Some of the risk factors have been already identified and confirmed: chronic hypertension, chronic renal disease, elevated body mass index, maternal age older than 40 years, nulliparity, anitphospholipid antibody syndrome, pregestational diabetes mellitus, preeclampsia in a previous pregnancy. Calcium supplementation reduces the risk of developing preeclampsia in high-risk women and those with low dietary calcium intakes. Low dose aspirin is effective for women at increased risk preeclampsia. Preeclampsia is characterized as mild or severe based on the degree of hypertension and proteinuria, and the presence of symptoms resulting from the involvement of the kidneys, brain, liver, and cardiovascular system. HELLP syndrome is a variant of severe preeclampsia characterized by hemolysis, elevated liver enzymes and low platelet count. HELLP syndrome occurs in up to 20 percent of pregnancies complicated by severe preeclampsia.

Common regimen for expectant management of mild preeclampsia consists of regular maternal and fetal monitoring. The decision to deliver involves balancing the risks of worsening preeclampsia against those of prematurity. Delivery is generally not indicated for women with mild preeclampsia until 37 to 38 weeks of gestation and should occur by 40 weeks. Patients with severe preeclampsia are admitted to the hospital, placed on bed rest and carefully monitored. The goals of treatment are to prevent seizures, lower blood pressure to avoid maternal end organ damage, and expedite delivery. Treatment of preeclampsia consists of magnesium sulfate, antihypertensive medications and fluid management. The use of magnesium sulfate helps to prevent seizures in women with preeclampsia. Its use is controversial in women with mild preeclampsia. Magnesium sulfate has the additional benefit of reducing the incidence of placental abruption. The optimal level of blood pressure control in pregnancies complicated by hypertension is unknown. Less tight control may decrease the

risk that the infant will be small for gestational age but it may increase the risk of respiratory distress syndrome of the newborn. Intravenous labetalol and hydralazine are commonly used for the acute management of preeclampsia. For women with severe preeclampsia undergoing expectant management remote from term, oral labetalol and nifedipine are acceptable options. Delivery is the only cure for preeclampsia. Most patients with preeclampsia respond promptly to delivery with decreased blood pressure, diuresis and clinical improvement. Eclampsia may occur postpartum, the greatest risk is within the first 48 hours. Magnesium sulfate is continued for 12 to 24 hours or occasionally longer if clinical situation warrants. There are no reliable data on postpartum hypertensive management; however oral nifedipine is commonly used (Leeman and Fontaine, 2008). The ethiology and pathogenesis of preeclampsia remain unclear, at present most investigators still recommend hydralazine for acute hypertension and methyldopa for treatment of chronic hypertension in pregnancy. Labetalol and nifedipine have been recently become also common in the treatment.

Importance of uterine denervation during pregnancy

During pregnancy, dramatic changes take place in all physiological functions both in mammals and humans, but the most impressive changes are restricted to the reproductive organs. One of these changes is the remodeling of uterine innervation, which is a well described but not completely understood, therefore intensively investigated phenomenon.

It is a known fact that the uterus of a non-pregnant rat has adrenergic (Adham and Schenk, 1969), cholinergic (Bell, 1972) and peptidergic (Papka *et al.*, 1985) innervation. These innervations not only affect the vessels of the myometrium but the smooth muscle of the myometrium itself (Zuspan *et al.*, 1981, Brauer *et al.*, 1992).

The extent of reinnervation is still under investigation and there is no clear answer for it. First it was thought that this denervation affects only adrenergic nerves (Thorbert *et al.*, 1979), but later it was proved that cholinergic and peptidergic fibres also undergo this denervation procedure (Hervonen *et al.*, 1973, Moustafa, 1988, Haase *et al.*, 1997). That this process is also present in other species than rats was also supported by other experimental data which showed e.g. the decrease of functional storage vesicles in the uterus of guinea pig at term pregnancy (Fried *et al.*, 1985) and this was also found in human uterus (Arkinstall and Jones, 1985).

As adrenergic system - besides prostaglandins and oxytocin - has a key function in regulating the contractility of pregnant uterus therefore all three systems are extensively investigated, and each system is targeted in tocolytic therapy (Giles and Bisits, 2007, Simhan and Caritis, 2007). We chose to examine adrenergic system since it is considered the most important in the regulation of uterine contractility and this is the system which is targeted routinely during tocolysis.

Sympathetic axons supplying the uterus reach the organ following different routes. Sympathetic nerves approach the uterus through the parametrial tissue. Some nerves travel in association with the uterine artery and its branches, as perivascular and paravascular bundles, while others travel free in the parametrial tissue as large nerve trunks and isolated nerve fibres. Within the uterus, sympathetic nerves innervate intrinsic blood vessels, generally forming a well-developed perivascular plexus, and distribute within the myometrium, usually following the direction of smooth muscle cells. The density of myometrial innervation shows considerable regional variations, being more developed in the tubal end of the uterine horn and cervix than in the main parts of the uterine horn/body (Owman *et al.*, 1967, Adham and Schenk, 1969). In the rat, the density of sympathetic nerves associated with the longitudinal myometrial layer is more developed in the cephalic than in the caudal region of the uterine horn, whereas the innervation of the circular myometrial layer is more developed in the caudal region (Zoubina *et al.*, 1998). The endometrium generally receives a reduced sympathetic innervation, which is mainly associated with the radial arteries.

During pregnancy both the vasculature and the smooth muscle is denervating, and it is slowly reinnervating after delivery (Haase *et al.*, 1997). Changes in the innervation during pregnancy are paralleled by a dramatic reduction in noradrenaline levels in the uterus, as well as in the activity of the noradrenaline synthesizing enzymes, tyrosine hydroxylase and dopa-decarboxylase (Owman *et al.*, 1975, Thorbert *et al.*, 1979). Reductions of noradrenaline levels were shown to result from degeneration of sympathetic terminal branches and not solely from a dilution of intact nerves in the enlarged uterus (Sporrong *et al.*, 1981). The functional significance of the degeneration of uterine sympathetic nerves during pregnancy still remains obscure. It is thought that this selective neurodegeneration may be necessary to protect foetus-placental circulation from ischemia, to maintain myometrial quiescence during the growth and accommodation of the foetus, and to prevent pre-term labour by reducing uterine contractility. The physiological decrease in uterine noradrenaline observed during normal pregnancy is disturbed in some pathological conditions, such as preeclampsia. In preeclampsia, the

myometrial concentration of noradrenaline and its co-transmitter the neuropeptide Y remains elevated at term and this is associated with preservation of sympathetic nerve fibres (Fried *et al.*, 1986, Rydhstrom *et al.*, 1989).

The degeneration of uterine sympathetic nerves along pregnancy is a complex phenomenon, which might involve at least three controlling factors, (1) the hormonal environment of pregnancy; (2) local influences from the foetus and placenta and (3) the mechanical stretching of the uterine wall produced by the growing foetuses. Based on the observation that pregnancy-induced sympathetic denervation is less severe in the empty than in the foetus-bearing uterine horn, it may indicate the contribution of mechanical stretching of the uterine wall to the degeneration of uterine sympathetic nerves at term (Lundberg *et al.*, 1989, Chavez-Genaro *et al.*, 2006). Progesterone has been also reported as playing key role in the degeneration of uterine sympathetic nerves during pregnancy. A causal relationship between high levels of progesterone in the uterus and reductions in noradrenaline in the organ has been described by Thorbert (Thorbert *et al.*, 1976). The role played by oestrogen in the degeneration of uterine sympathetic nerves during pregnancy still remains unclear.

Sympathetic innervation is restored after delivery, however the density of innervation observed in the uterus of the virgin female is never re-established after the first pregnancy. In addition the rate of recovery presents considerable regional and inter-species differences. It was also revealed that pregnancy-induced sympathetic denervation of the uterus also involves Schwann cells (Lundberg *et al.*, 1987). Since Schwann cells provide soluble and substrate associated factors necessary for the regeneration of the nerves, recovery of innervation following pregnancy is slower than following chemical sympathectomy with 6-hydroxydopamine, which does not affect Schwann cells. Restoration of the normal innervation in the empty uterine horn and cervix is faster than in the fertile horn because nerves remain largely structurally intact.

The degree of degeneration of the extrinsic uterine sympathetic innervation in the parametrial tissue also appears to contribute to the differential regional rate of nerve regeneration observed between the fertile and empty uterine horns. It was revealed in guinea pigs, that extrinsic nerves in the parametrial tissue adjacent to the foetus-containing uterine horn also undergo pronounced degenerative changes, comprising both axons and Schwann cells (Alm *et al.*, 1988). These degenerative changes are not observed in the parametrial tissue adjacent to the empty uterine horn. In the rat uterus, the innervation of parametrial tissue remains largely intact at term (Bianchimano *et al.*, 2007) and uterine sympathetic nerves consistently

regenerate a few days following delivery (Haase *et al.*, 1997, Klukovits *et al.*, 2002). Contrary to this, restoration of innervation takes several weeks in the foetus-containing uterine horn of the guinea pig because physical restoration of nerves is involved. In the rat the denervation of intrauterine blood vessels is not complete at term and these nerves may contribute to rapid restoration of innervation after delivery.

Ultrastructural characterization of pregnancy-induced denervation has been performed mainly using immunohistochemical methods (Alm *et al.*, 1988, Haase *et al.*, 1997). By using these techniques the denervational process throughout pregnancy can be described and the structural-morphological differences in the denervation of the myometrium and myometrial vessels can also be determined, but these techniques are not able to answer the question how the functionality of these neurons changes during pregnancy. Considering above mentioned facts it sounded reasonable that the deterioration of function is initiated earlier than the structural loss could be detected, therefore functional approach may provide a more detailed insight into this process.

Some studies investigate the pharmacological reactivity of uterus and describe the adrenergic receptor status during pregnancy which can be also considered as a functional approach (Engstrom *et al.*, 1997). However the results of these methods are also influenced by numerous other factors which also change during pregnancy, like the density and affinity (Dahle *et al.*, 1993, Kaneko *et al.*, 1996, Adolfsson *et al.*, 1998) and the coupling to signal transductional mechanisms of the targeted receptors (Mhaouty *et al.*, 1995).

After considering the above mentioned facts in investigating pregnancy-induced denervation in rats, our aim was to set up a novel experimental approach which is suitable for investigating this process from a purely functional view, not only during pregnancy but in the postpartum period which is less frequently examined.

We hypothesized that the loss of function of adrenergic nerves may be detected earlier than the structural changes by immunohistochemical methods. It was also our aim to determine the recovery of function after delivery and to compare these findings to immunohistochemical results. In the selected experimental system, we are able to measure neurotransmitter concentration only, instead of morphological parameters, but from the dynamics of neurotransmitter liberation and uptake we can conclude the function and integrity of the neurons. As the presynaptic uptake and stimulated release of the transmitter can be regarded as a functional marker of the adrenergic nerves, superfusion technique was chosen.

It has been 30 years since Raiteri and his colleagues assembled the first superfusion device, which has not changed much since then. With this method the presynaptic release of several neurotransmitters can be determined. Initially it was designed to investigate the transmitter reuptake inhibiting or release enhancing effect of several pharmacons (Raiteri *et al.*, 1974). The principle of superfusion technique is that the released transmitter (either it is released spontaneously or elicited by a stimulus) is carried away from the site of release with the help of a constant flow, inhibiting that the released transmitter would get back into the neuron with reuptake mechanism. The method is mainly used in neurological experiments for the functional investigation of the presynaptic side of transmissions. Using superfusion technique improved our knowledge about neurotransmission, it was found e.g. that certain neurotransmitters can be released not only with exocitosis but with carrier mediated transport (Raiteri *et al.*, 1979). This technique is also frequently used for the investigation of mechanism of action of agents acting in the central nervous system (Gerevich *et al.*, 2001).

With this method, the electrically evoked release of [^{3}H]noradrenaline was investigated as a function of gestational age and postpartum period.

Since histological data showed that the cervical adrenergic innervation remains intact or little affected and there is only limited data available on the functional changes of cervix during pregnancy, cervical samples were also investigated by superfusion technique (Bryman *et al.*, 1987, Lundberg *et al.*, 1987, Norstrom and Bryman, 1989).

After investigating and exploring functional changes in the adrenergic innervation of both uterus and cervix during pregnancy, we decided to investigate how could denervation be influenced from a pharmacological point of view and if our technique is suitable for this or not.

Our scope turned to investigate the effects of α-methyldopa, which is a widely used drug in pregnancy, but its mechanism of action is not completely understood. We planned to investigate how α-methyldopa acts on adrenergic neurons and what are the postsynaptic consequences of this effect.

Since hypertension disorders are one of the most important complications of pregnancy, affecting approximately 10% of all pregnancies and contributing greatly to maternal and perinatal morbidity and mortality throughout the world (Frishman *et al.*, 2005), and α-methyldopa is one of the most frequently used antihypertensive agent in preeclampsia, gestational hypertension and chronic hypertension in pregnancy (Sibai, 1996, Magee, 2001,

Borghi *et al.*, 2002, Gunenc *et al.*, 2002), we have decided to investigate its effect on the pregnancy-induced adrenergic denervation of rat uterus.

α-methyldopa is a centrally acting antihypertensive drug, which enters the biosynthesis of catecholamines (Henning and Rubenson, 1971, Day *et al.*, 1973, Finch and Haeusler, 1973), and is metabolized into α-methylnoradrenaline, which is a potent α_2-adrenergic agonist (Goldberg *et al.*, 1982). Thereby α-methyldopa decreases the sympathetic activity of the autonomic nervous system. Its metabolites bind mainly to α_2-adrenergic receptors, but have affinity for β_2 and α_1-adrenergic receptors (Goldberg *et al.*, 1982). Since the antihypertensive effect of α-methyldopa is mediated via adrenergic mechanisms which basicly determines the myometrial contractility (Thorbert *et al.*, 1979). Furthermore the fact that the information on the myometrial action of α-methyldopa is very limited, supported our aim to investigate its effects on myometrial noradrenaline release and contractility.

We have also decided to try to explore the effects of α-methyldopa on pregnant mymetrium in depth, therefore we have performed isolated organ experiments to determine the sensitivity of α-methyldopa treated uterus to α and β adrenergic receptor agonists. We hypothesized if α-methyldopa has an effect on the adrenergic neuron in this case the adrenergic receptors will receive different stimulation pattern, which may alter their affinity, number or coupling. Radioligand binding technique was also used to assess if the changes caused by α-methyldopa treatment in the uterus contractility correlate with direct receptorial changes or other signal transductional pathways can also be responsible for the contractility alterations of α-methyldopa treated uterus.

Materials and Methods

Experimental animals

Sprague–Dawley rats (200–250 g for females) were mated in a special cage in the early morning; copulation was determined by the presence of a copulation plug or sperm in a native vaginal smear. The day of conception was considered to be the first day of pregnancy. Rats were killed by cervical dislocation. Non-pregnant rats used in the experiments were all virgin animals in the estrus state of their cycle.

In the α-methyldopa treatment experiments the animals in the active group were treated with daily 200 mg/kg α-methyldopa intraperitoneally for 7 days and the experiment was performed on the 7^{th} day. Control animals were treated with physiological saline.

All experimental animal protocols satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the University of Szeged.

Release of [^{3}H]noradrenaline

Samples of uterine and cervical tissue (20–30 mg) were dissected; the samples from the implantation and inter-implantation sites were processed separately. Myometrial samples were cleared from connective tissue and endometrium. The wet weights of the samples were measured, they were minced and incubated with 10^{-7} M [^{3}H]noradrenaline at 37°C for 60 min. The samples were then washed three times with de Jongh buffer, and the pieces were placed into superfusion chambers (Experimetria, Budapest, Hungary), which were superfused continuously for 60 min at a flow rate of 1 ml/min with de Jongh buffer containing the monoamine oxidase (MAO) inhibitor pargyline, the noradrenaline-reuptake inhibitor desipramine and the extraneuronal reuptake inhibitor deoxycorticosterone (each 10 μM). (Sugimori T *et al*., 1987) During the experiments carried out with lidocain, the concentration of lidocain in the buffer was 50 μM. The composition of the de Jongh buffer was 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4 and 6 mM glucose, pH 7.4. The solution was maintained at 37°C and equilibrated throughout the experiment with O_2 containing 5% (v/v) CO_2. (Raiteri *et al.*, 1974) After a 60-min wash-out period, a total of 22 3-min fractions were collected. At the end of the experiment, the tissue samples were solubilized in 1ml Solvable (Canberra-Packard, Budapest, Hungary) for 3 h at 60°C. The [^{3}H] content in each 3-min fraction and tissue solution was determined with a liquid scintillation spectrometer. Electric field stimulation (EFS) consisting of squarewave

pulses was applied to the tissues, using a programmable stimulator (Experimetria, Budapest, Hungary). EFS was applied twice after the wash-out period, during fractions 5 and 15. Each period of stimulation consisted of 360 pulses (voltage, 40 V; pulse width, 2 ms; frequency, 2 Hz; these parameters are suitable for selective neural stimulation). The [^{3}H] noradrenaline contents in the fractions were expressed as fractional release. This is the amount of labelled transmitter liberated during a 3-min fraction as a percentage of the actual radioactivity content in the tissue at the time of sampling. Peak releases were calculated by subtraction of the radioactivity of the fourth and fourteenth fractions from that of the fifth and fifteenth fractions, respectively. The tissue activity (expressed in dpm/mg tissue) was used to describe the uptake capacity of the sample for [^{3}H]noradrenaline. Differences between mean values of denervation and reinnervation experiments were evaluated by using one-way analysis of variance (ANOVA) with Dunnett's post hoc test. Differences between implantation and inter-implantation sites were evaluated by using the unpaired t test. Data from the α-methyldopa superfusion experiments were evaluated by one-way ANOVA followed by Newman-Keuls post test.

Statistical analysis of the data was performed with GraphPad Prism 2.01 (Graph Pad Software, San Diego, CA, USA). All reported data are mean results from at least six independent experiments.

Isolated organ studies

Uterine rings were taken from the uterine horns of pregnant or non-pregnant, treated or non-treated rats. Two muscle rings were sliced from both horns of the uterus and mounted vertically in a tissue bath containing 10 ml de Jongh buffer (see composition in Release of [^{3}H] noradrenaline section). (Jerde *et al.*, 1999) The temperature of the tissue bath was set to and maintained at 37°C, and O_2 containing 5% (v/v) CO_2 was perfused continuously through the bath. Tissue samples were equilibrated under these conditions for 90 min before the experiments were started. The initial tension of the uterus rings was set to 1.5 g, which dropped to approximately 0.5 g by the end of the equilibration period. The tensions of the myometrial rings were measured with a strain gauge transducer (SG-02, Experimetria, Budapest, Hungary) and recorded with an Isosys Data Acquisition System (Experimetria, Budapest, Hungary). The areas under the curves were analyzed for a 5-min period after each administration of the tested substances. (Gáspár *et al.*, 1998)

Determination of contractility changes

Cumulative dose-response curves were constructed for noradrenaline in the concentration range $1x10^{-10}$ – $1x10^{-5}$ M. The chamber contained propranolol (10^{-6} M) to block the relaxation component mediated by β-adrenergic receptors. During equilibration period (90 minutes), buffer in the chambers was changed every 15 minutes, totally 6 times. After equilibration, noradrenaline was added to the chamber cumulatively, in a total of 11 different concentrations and the contractility answers were recorded for 5 minutes after each administration. At the end of the experiment, KCl (70 mM) was added to the chamber and the evoked contractions were considered as maximal tone and recorded also for 5 min. The contractions induced by noradrenaline were expressed as a percentage of the KCl evoked contractions.

To characterize the effects of α-methyldopa on the β-adrenergic receptor-mediated myometrial relaxation, cumulative dose-response curves were additionally constructed for terbutaline. The experimental design was similar to the previous one, but the chamber did not contain propranolol. The terbutaline concentration range was 10^{-8} – 10^{-5} M (altogether 7 concentrations). KCl (50 mM) was added to the chamber before the start of the experiment in order to elicit an initial tension of the uterine rings which was regarded as 100% of the motor activity.

A sigmoidal curve was fitted individually to all dose-response curves (both noradrenaline and terbutaline) and the maximal effect and EC_{50} values were calculated by means of GraphPad Prism 2.01. To calculate the differences between mean values of the EC_{50} and E_{max} of the isolated organ experiments we have used two-way ANOVA followed by Bonferroni post test.

Radioligand-binding studies

Membrane preparation

Pregnant and non-pregnant Sprague-Dawley rats were killed with cervical dislocation. Uterine tissues were dissected carefully and the embryos were rapidly removed. All subsequent steps were performed at 4 °C. Uteri were homogenized in 6–10 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose with an Ultra Turrax 25 homogenizer (IKA Labortechnik, Staufen, Germany). The homogenate was centrifuged at 20,000× g for 10 min and the pellet was recentrifuged. The supernatants were collected and centrifuged at 50,000× g for 60 min and the pellet was used for saturation experiments. The

membrane preparation was stored at –70 °C until assayed. The protein concentration of the membrane fraction was measured by the method of Bradford with bovine serum albumin as standard (Bradford, 1976).

Saturation binding experiments

The saturation binding experiment is an appropriate assay to determine the equilibrium dissociation constant, K_d, and the maximal number of binding sites, B_{max}, of a radioligand. It was performed by incubating the cell membrane fraction with a range of concentrations of [^{3}H]RX 821002 (0.2-8.0 nM, α_2-adrenoceptors) and [^{3}H]ICI 118,551 (0.5-10 nM, β_2-adrenoceptors) at 25 °C for 45 min. At the end of the incubation, the bound radioligand was separated from the residual free radioligand by rapid filtration on a Brandell cell harvester through Whatman GF/C filters and washed with 3 × 10 ml of ice-cold buffer (Tris-HCl, pH = 7.41). The bound radioactivity was determined in a HighSafe scintillation cocktail in a Wallac 1409 liquid scintillation counter. The observed total binding consists of specific binding to the receptor itself, plus nonspecific binding to nonreceptor sites. The nonspecific binding was measured with 10 μM unlabeled yohimbine and alprenolol. The specific binding was calculated as the difference between the total and the nonspecific binding, and was plotted as a function of the free radioligand concentration. The K_d and B_{max} values were calculated with GraphPad Prism 2.01 software.

All assays were carried out at least three times in duplicate, and values are given as means ± SEM. Binding capacity (B_{max}) and equilibrium dissociation constants (K_d) of [^{3}H]RX 821002 and [^{3}H]ICI 118,551 were calculated according to Rosenthal (Rosenthal, 1967). The data from the radioligand-binding experiments were analyzed by using the GraphPad Prism 2.01 software, utilising a non-linear regression analysis.

Drugs

Pargyline, desipramine, deoxycorticosterone, noradrenaline, terbutaline, propranolol, yohimbine, lidocain and alprenolol were purchased from Sigma-Aldrich (Budapest, Hungary). (2)-7-[^{3}H](N)-Noradrenaline hydrochloride (specific activity, 7.94 Ci/mmol) was from Perkin Elmer Life Sciences (Boston, MA, USA), [^{3}H]RX 821002 (specific activity, 50 Ci/mmol) from Amersham Bioscience (UK), and [^{3}H]ICI 118,551 (specific activity, 18.8 Ci/mmol) from Tocris (UK).

Results

Results of sodium channel blockade on EFS-induced noradrenaline release

In order to confirm that the EFS-induced noradrenaline release is sensitive to sodium channel blockade, 50 μM lidocaine was added to the buffer used for perfusion.

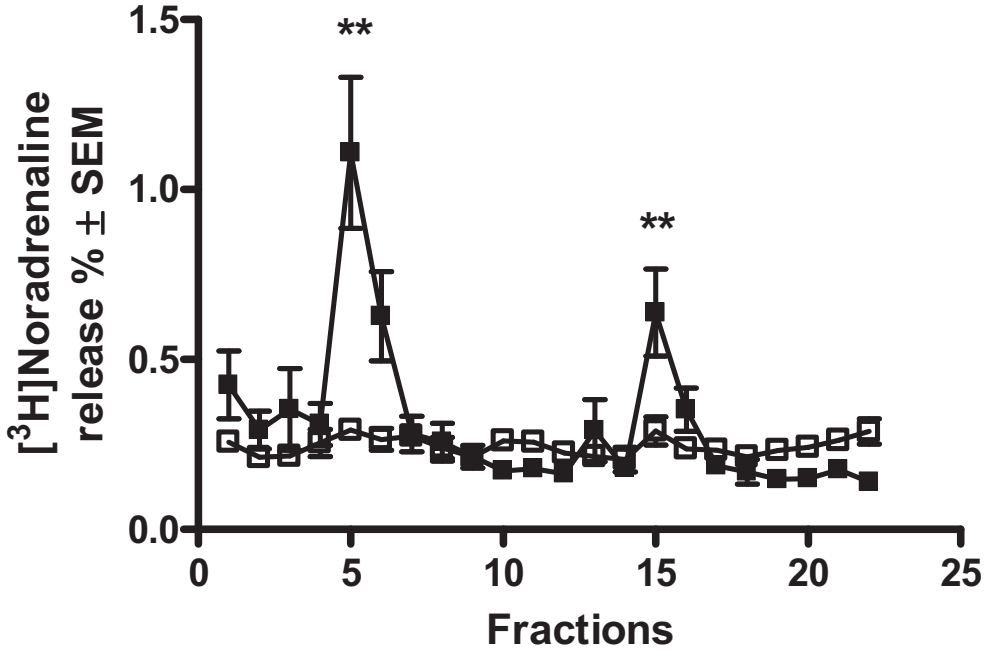

Figure 1. EFS-evoked fractional [^{3}H]noradrenaline release from myometrial samples at oestrus (control ■), and when 50 μM lidocain was added to the buffer (□).

As it can be seen in Figure 1 lidocaine completely abolished the electrically evoked noradrenaline release which supports our hypothesis that electric field stimulation with the current parameters evokes noradrenalin release selectively from neuronal elements.

Results of tissue activity determination

We used tissue activity to describe the [^{3}H]noradrenaline uptake capacity of the sample. It was found that tissue activity was highest in the non-pregnant state both in myometrial and cervical tissue (Figures 2 and 3).

In early pregnancy, on day 7, transmitter uptake was significantly lower for implantation sites than for the inter-implantational part of myometrium. The difference in denervation of the implantational and inter-implantational sites diminished by mid-pregnancy (Day 14), no site-specific difference could be detected in later stages of pregnancy and in postpartum period

either. This decline in labeled neurotransmitter uptake reached its minimum at the end of pregnancy. As far as the postpartum period was concerned, tissue activity of the uterus at both the implantation and inter-implantation sites remained lower than before pregnancy throughout the 28 days of the investigation. In contrast with the period of pregnancy, no site-dependent difference was observed in the myometrium. However, when the postpartum results were compared with the day-21 results, the myometrial samples displayed significantly higher values after 14 days, which points to a slow but detectable reinnervation procedure in uterus.

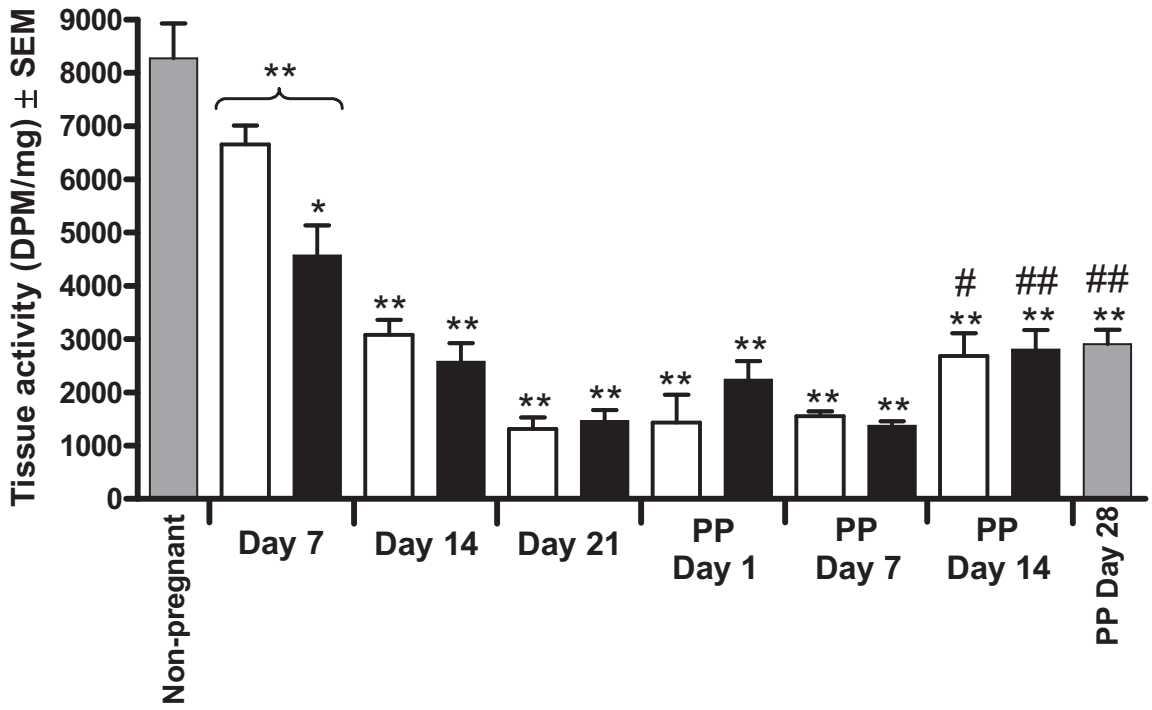

Figure 2. [^{3}H]Noradrenaline-uptake capacity of myometrium during gestation and the postpartum (PP) period. * and ** denote P < 0.05 and P < 0.01 as compared with the non-pregnant value, respectively; # and ## denote P < 0.05 and P < 0.01 as compared with the day-21 values, respectively. Black bars, implantation sites; white columns, inter-implantation sites (distinction between the two sites is not possible 28 days after delivery).

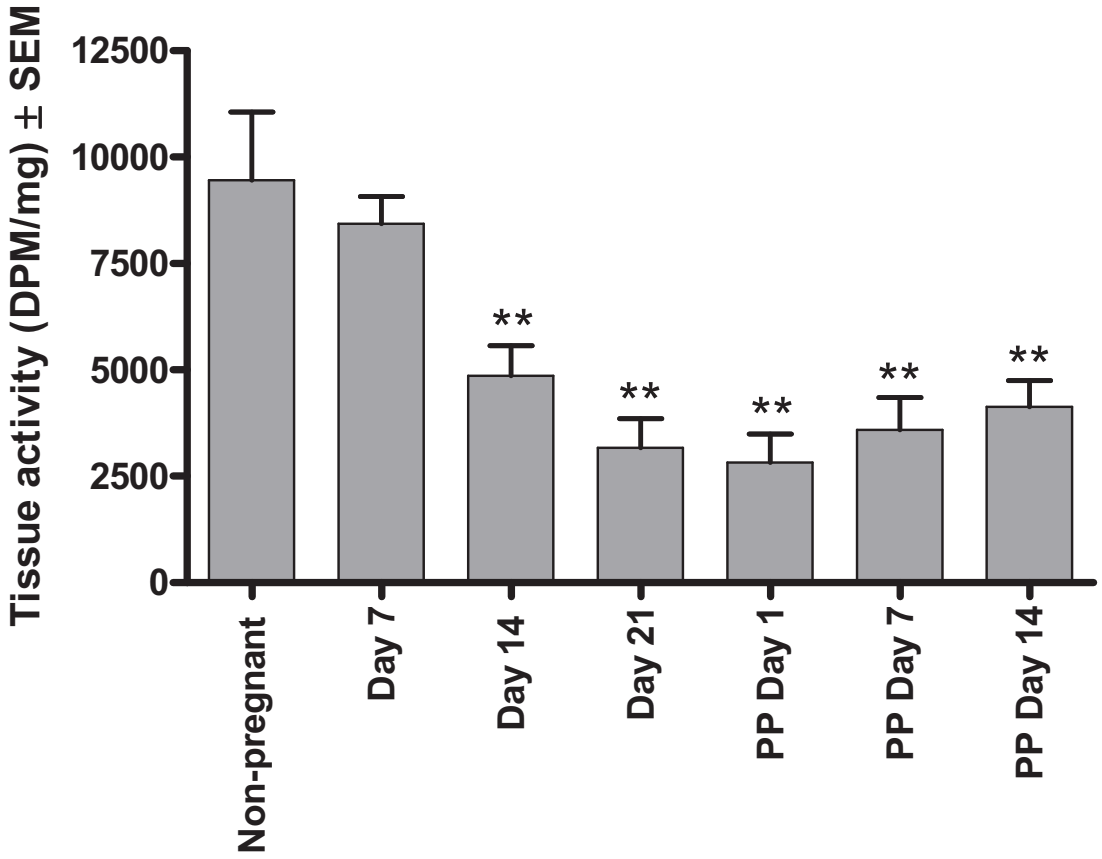

Figure 3. [^{3}H]Noradrenaline-uptake capacity of cervical tissue during gestation and the postpartum (PP) period. ** denote $P < 0.01$ as compared with the non-pregnant value

In non pregnant state uterus and cervix has similar tissue activity, but during pregnancy the decrease of tissue activity in cervix is less pronounced than in uterus. However, cervical tissue showed a similar trend, although decrease in uptake capacity was not significantly lower on Day 7 comparing to non-pregnant state, but by Day 14 this decrease resulted in a significantly lower tissue activity. Activities of the postpartum cervical samples up to day 14 were not significantly higher than the day 21 value.

Results of stimulated [^{3}H]noradrenaline release

EFS evoked a substantial [^{3}H]noradrenaline release in both the uterus and the cervix excised from non-pregnant rats (Figures 4 and 6). We have applied two stimulations in order to obtain information on the release capacity of the examined tissues. The second stimulus evoked a smaller neurotransmitter release comparing to the first peak. In myometrial tissue, a gradual decrease was detected in the peak evoked by EFS during the gestation. The amount of [^{3}H]transmitter released became less and less as the pregnancy progressed, and a gradual, but

not so extensive increase could be measured during the first 28 days of the postpartum period (Figure 5). Generally, it was found that the second peak elicited by EFS decreased in a more sensitive way than did the first one. This decrease was already significant on Day 7 for samples from implantational sites, but by Day 14 the quantity of the released neurotransmitter elicited by the second EFS, continued to decrease significantly both in samples from implantational and inter-implantational sites. Released neurotransmitter amount of first peak also showed significant reduction on Day 7 for implantational sites (Figure 4B) and only on Day 21 for inter-implantational sites (Figure 4D). A substantial and significant difference was found between the implantation and inter-implantation sites of the uterus in the early pregnant state, indicating that the loss of the adrenergic nerve function starts in the implantation area.

In the postpartum period, a gradual increase in the function of the noradrenergic nerves could be demonstrated in the uterus, but even after 28 days the EFS induced release was still less than in non-pregnant state, and the stimulated release of [^{3}H]noradrenaline was not significantly different from that on the last day of pregnancy. In contrast with the denervational process, reinnervation could be detected only in the first peak. It is assumed that the first EFS exhausted the limited transmitter capacity of the regenerating nerves, resulting in a decreased second peak.

As concerns the cervical samples, a gradual tendency of the EFS-evoked [^{3}H]noradrenaline release to decrease was observed during pregnancy, but these changes were not significant statistically (Figure 6).

The EFS-evoked release of [^{3}H]noradrenaline from the cervical samples was significantly suppressed in the early postpartum period, and approximated the non-pregnant level 14 days after delivery (Figure 6). Cervical adrenergic reinnervation was followed only up to postpartum day 14.

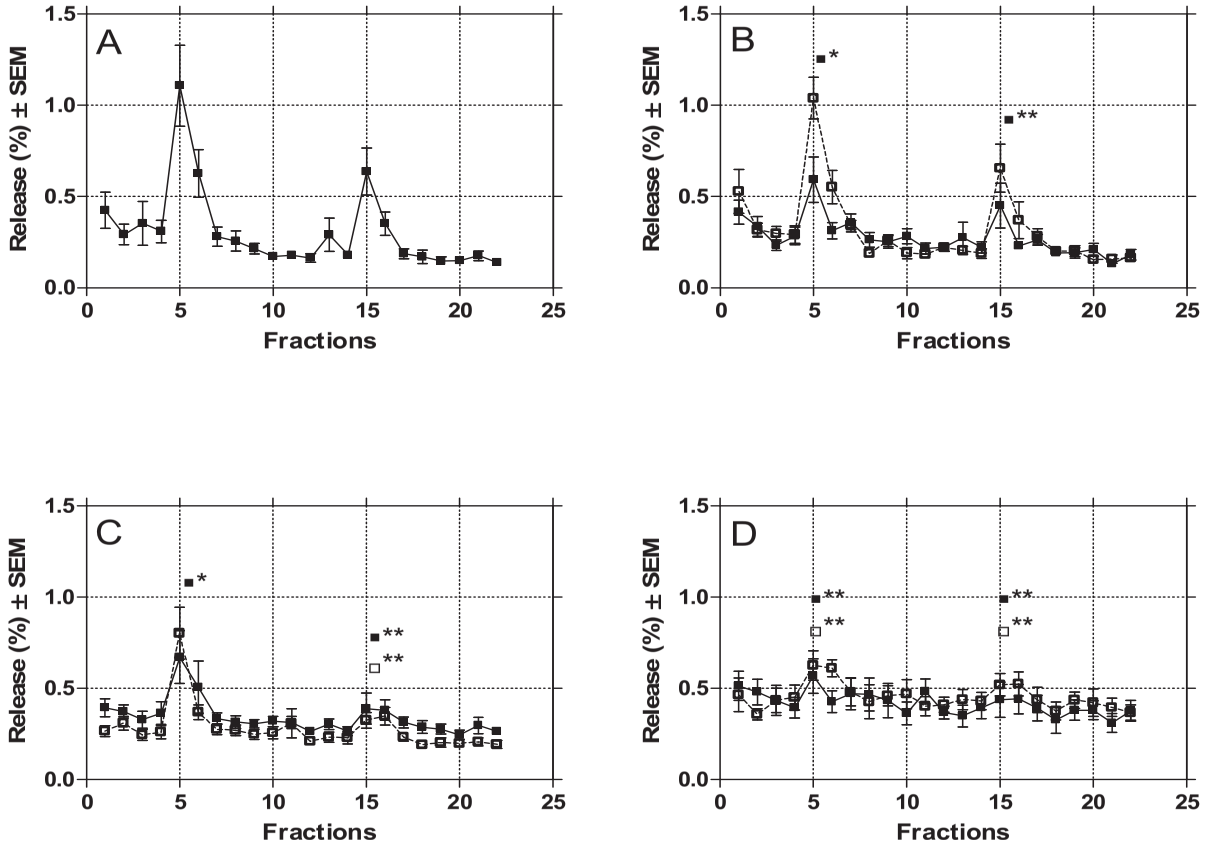

Figure 4. EFS-evoked fractional [^{3}H]noradrenaline release from myometrial samples at oestrus (A), and on days 7 (B), 14 (C) and 21 (D) of pregnancy. * and ** denote $P < 0.05$ and $P < 0.01$ as compared with the non-pregnant value, respectively. During gestation, ■ and □ indicate release from the implantation and inter-implantation sites, respectively.

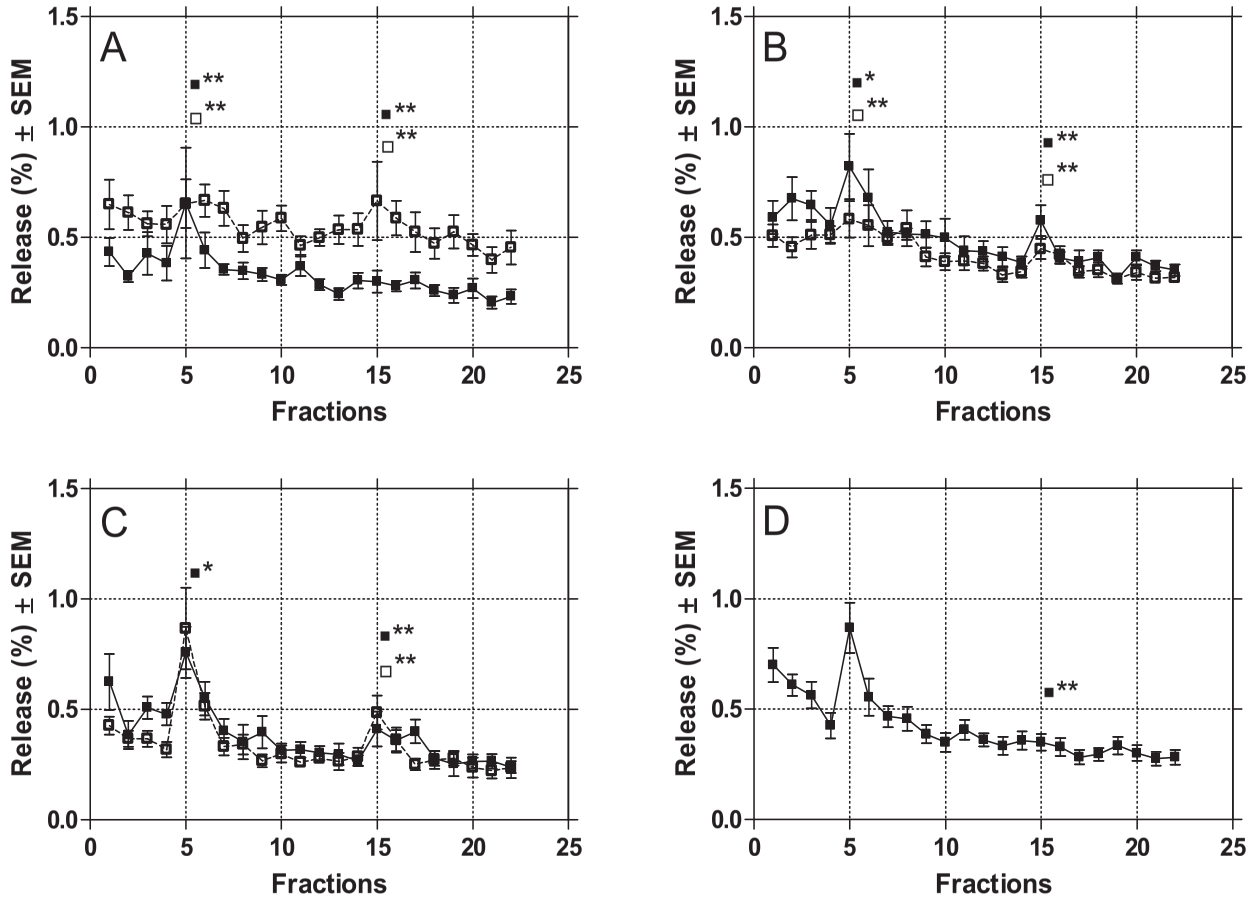

Figure 5. EFS-evoked fractional [^{3}H]noradrenaline release from postpartum myometrial samples on days 1 (A), 7 (B), 14 (C) and 28 (D). * and ** denote P < 0.05 and P < 0.01 as compared with the non-pregnant value, respectively. In A–C, ■ and □ indicate release from the implantation and inter-implantation sites, respectively (distinction between the two sites is not possible 21 days after delivery)

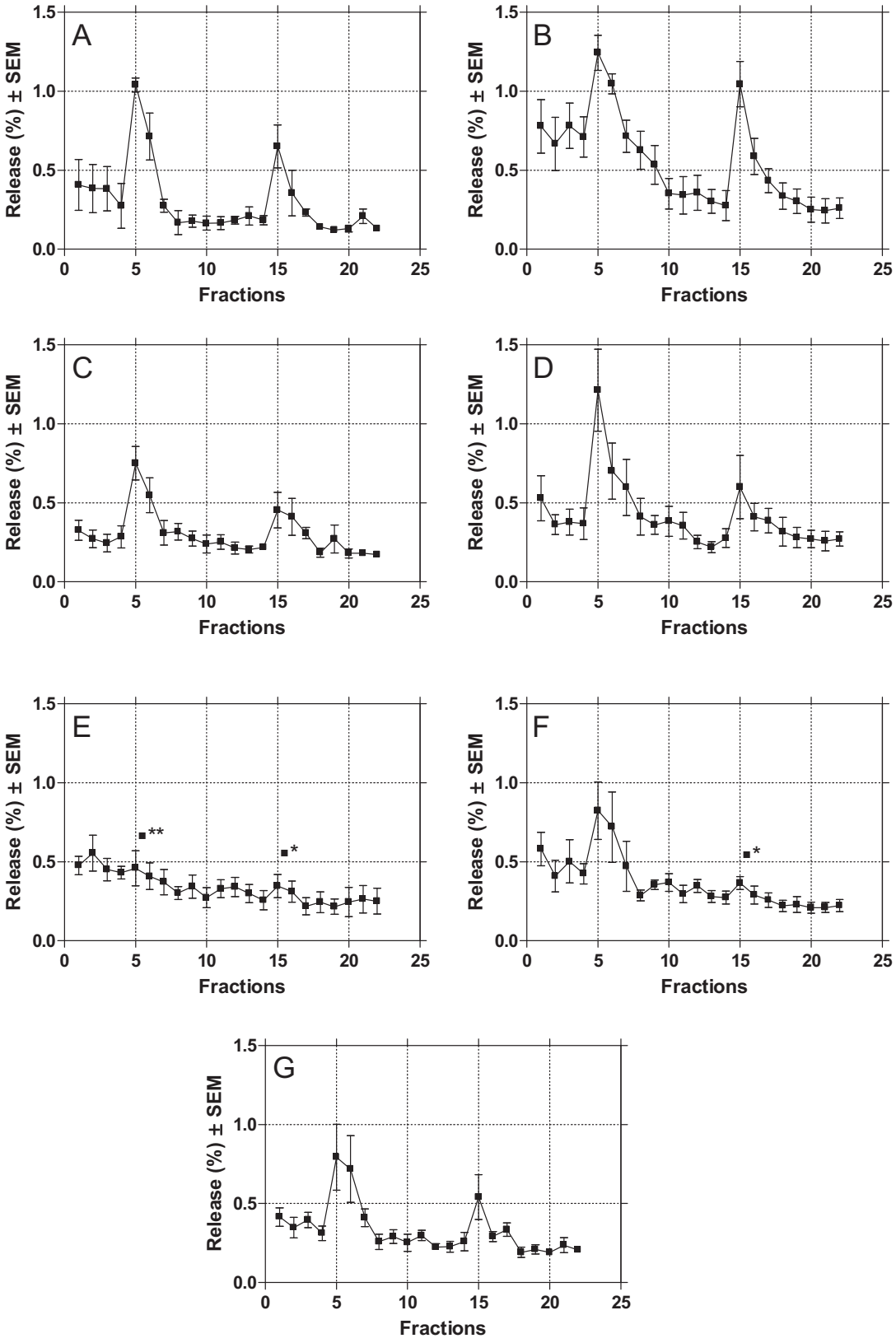

Figure 6. EFS-evoked fractional [^{3}H]noradrenaline release from cervical samples at oestrus (A), on days 7 (B), 14 (C) and 21 (D) of pregnancy, and on postpartum days 1 (E), 7 (F) and 14 (G). * and ** denote $P < 0.05$ and $P < 0.01$ as compared with the non-pregnant values, respectively.

Results of experiments concerning the myometrial effect of α-methyldopa

Tissue radioactivity determination

The tissue radioactivity (expressed in dpm/mg) was used to describe the uptake capacity of the sample for [^{3}H]noradrenaline (Figure 7) as in the previous experiments.

Treatment with α-methyldopa (daily 200 mg/kg intraperitoneally for 7 days) decreased the amount of labeled noradrenaline in both non-pregnant and early pregnant animals. Although the difference between the tissue radioactivities from animals in term were also relevant but statistically not significant compared to the non-treated tissues.

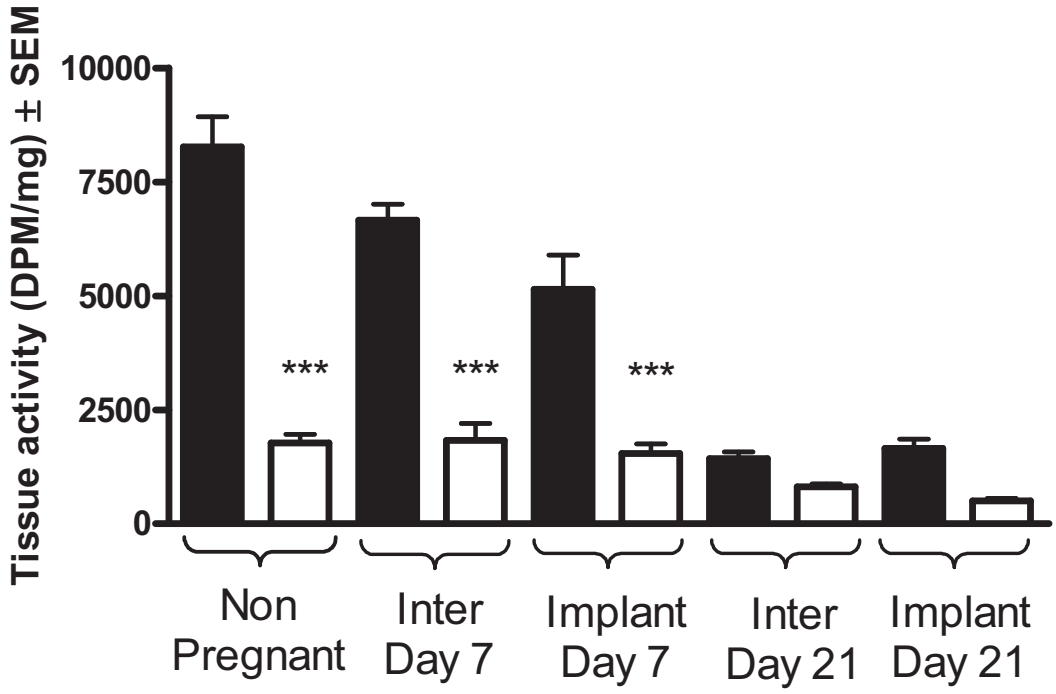

Figure 7. The effects of α-methyldopa treatment on the [^{3}H]noradrenaline-uptake capacity of myometrial samples from non-pregnant and from 7-day and 21-day pregnant rats. * denote P<0.001 as compared with the nontreated value. ■ indicates control values, and □ indicates α-methyldopa-treated values.**

Results of stimulated [^{3}H]noradrenaline release of α-methyldopa

Electric field stimulation (EFS) evoked a substantial [^{3}H]noradrenaline release in the uterus isolated from non-pregnant rats (Figure 8). For the α-methyldopa-treated non-pregnant rats, this EFS-evoked [^{3}H]noradrenaline release was almost completely abolished. On day 7 of pregnancy the [^{3}H]noradrenaline release was decreased at both implantation and inter-implantation sites due to the pregnancy-induced adrenergic denervation. α-Methyldopa treatment substantially decreased the first EFS-evoked [^{3}H]noradrenaline release at both sites, while the second peak proved significant only at the inter-implantational sites. This is probably due to the fact that pregnancy-induced adrenergic denervation is more pronounced at implantational sites, so the release decreasing effect of α-methyldopa is not so remarkable. At the end of pregnancy (day 21) the amount of [^{3}H]noradrenaline released from the α-methyldopa-treated animals was not changed as compared with the control level. The elevation of the baseline release at the implantation site is a consequence of the way of the calculation of fractional release: a decreased [^{3}H]noradrenaline uptake by the treated tissues results in a relatively higher liberation during a 3-min period when given in percentage.

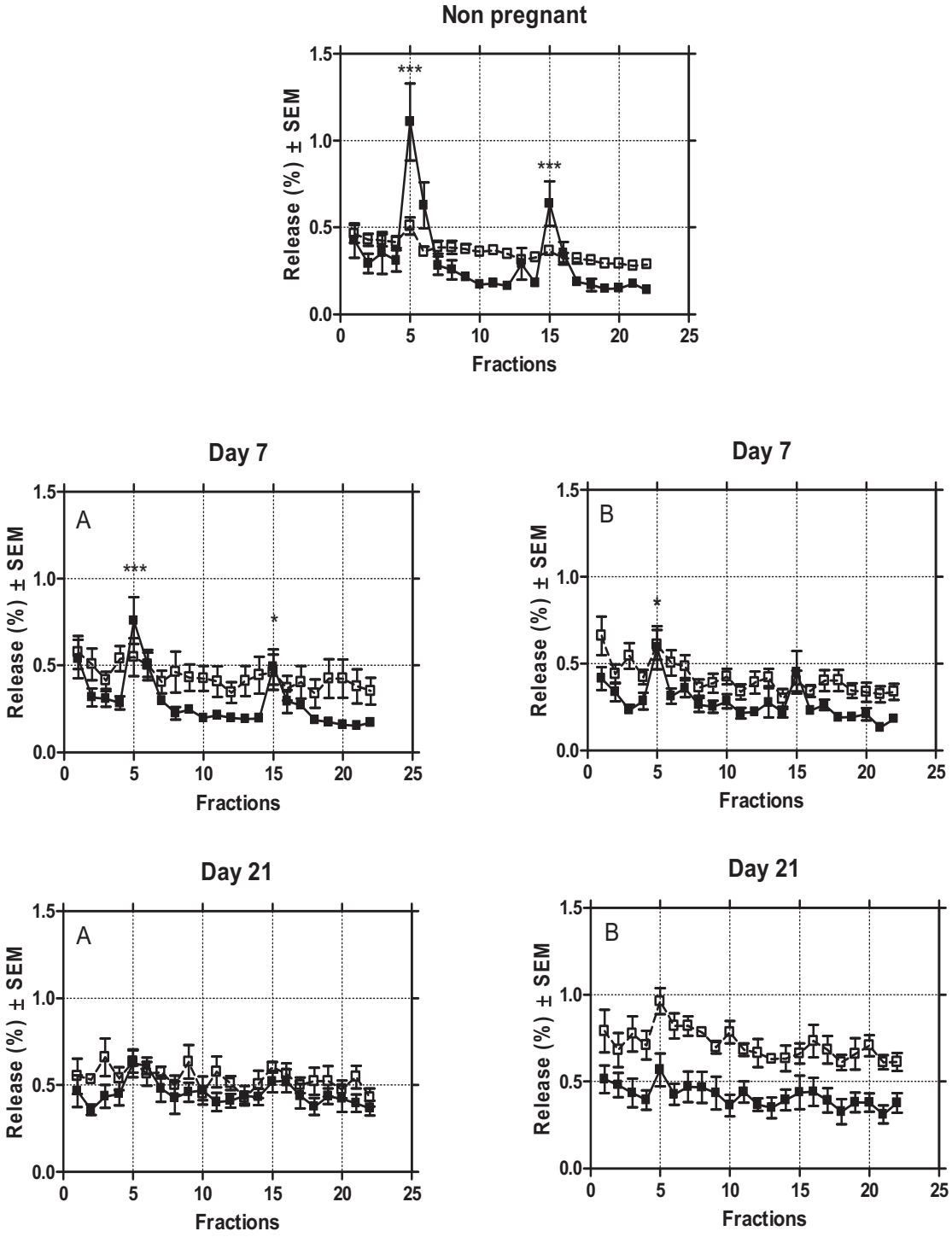

Figure 8. The effects of α-methyldopa treatment on the EFS-evoked fractional [^{3}H]noradrenaline release from myometrial samples at estrus (upper panel), on day 7 (middle panels) A – at inter-implantational sites B – implantational sites and on day 21 (lower panels) A – inter-implantational sites B - at implantational sites . * and * denote P<0.05 and P< 0.001 as compared with the nontreated value, respectively. ■ indicates control values, and □ indicates α-methyldopa-treated values.**

Results of isolated organ experiments

Noradrenaline did not have any effect on the non-pregnant uterus and the α-methyldopa-treated non-pregnant uterus exhibited a higher spontaneous activity without being more sensitive to α-adrenergic stimulation (Figure 9, Table 1). This higher spontaneous activity of the α-methyldopa-treated non-pregnant uterus can be seen in Figure 9A, where the noradrenaline elicited contractions are more intensive at the smallest dose applied already. On day 7 of pregnancy, noradrenaline evoked weak dose-dependent contractions in the control group, but more profound contractions in the uteri of α-methyldopa-treated rats (Figure 9B). At term pregnancy (day 21), noradrenaline increased the contractions in a dose-dependent manner. There was no difference in the noradrenaline-evoked contractions between the control and the α-methyldopa-treated group on day 21.

In the next set of experiments, we examined the myometrial relaxing effect of terbutaline, we found that terbutaline inhibited the KCl elicited contractions in a dose-dependent manner (Figure 10, Table 2). α-Methyldopa treatment shifted the dose-response curve of terbutaline slightly to the right and decreased its maximal effect significantly in the non-pregnant animals. On day 7 of pregnancy, α-methyldopa treatment resulted in a higher maximal effect. At term, α-methyldopa treatment significantly and substantially decreased the relaxant effect of terbutaline, as evidenced by a decreased maximal effect and a higher EC_{50} value.

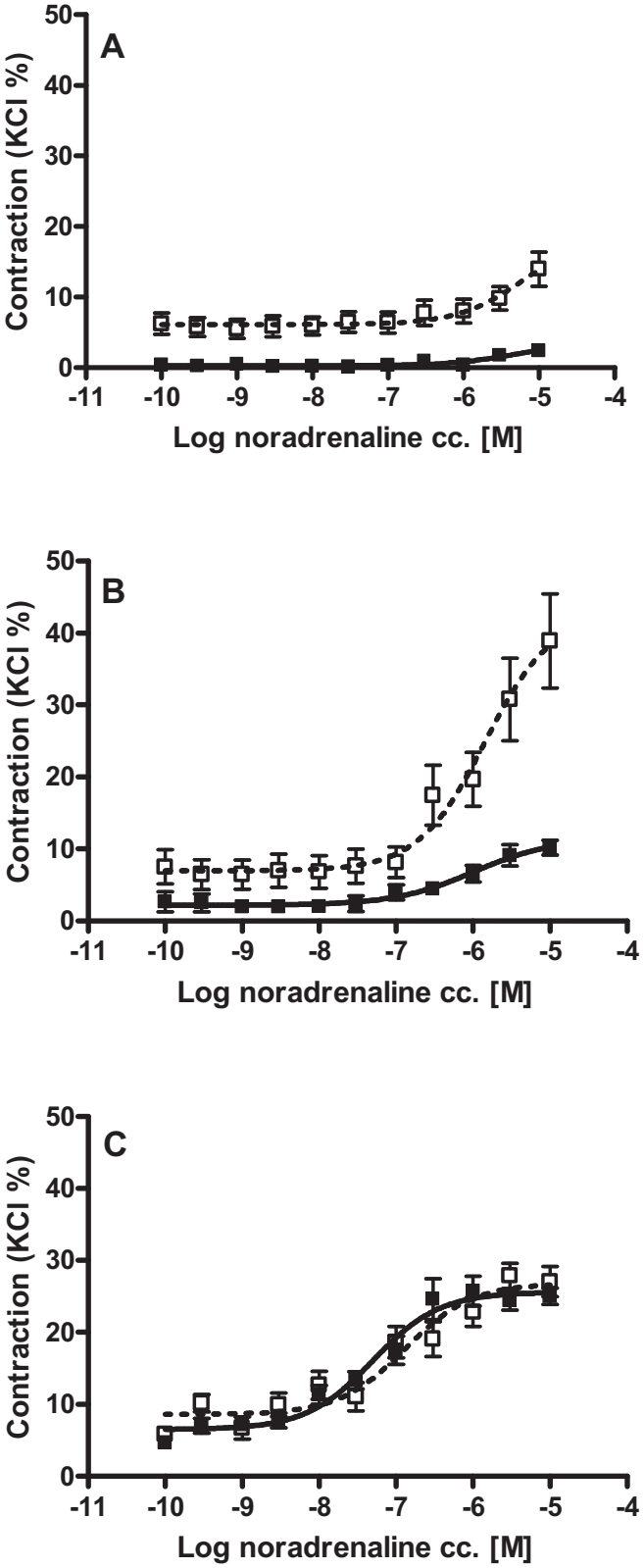

Figure 9. The effects of α-methyldopa treatment on the myometrial contractility evoked by noradrenaline at estrus (A), and on day 7 (B) and on day 21 (C). ■ indicates control values, and □ indicates α-methyldopa-treated values.

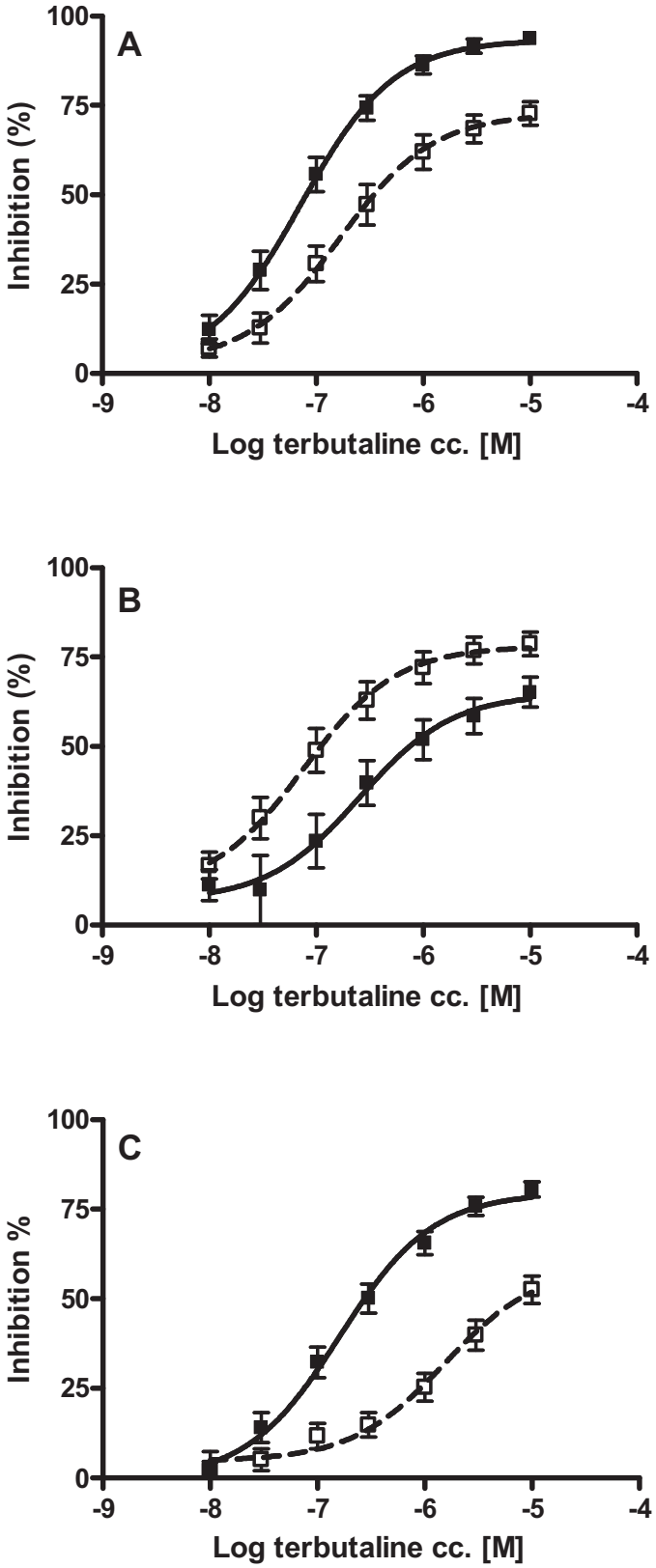

Figure 10. The effects of α-methyldopa treatment on the relaxation effect of terbutaline at estrus (A), and on day 7 (B) and on day 21 (C). ■ indicates control values, and □ indicates α-methyldopa-treated values.

Table 1. Calculated parameters of the dose response curves generated by α-adrenergic stimulation (Figure 9).

	Control			α-Methyldopa treated			p value (Emax)	p value (EC50)
	Emax ± SEM (%)	n	EC50 ± SEM (μM)	Emax ± SEM (%)	n	EC50 ± SEM (μM)		
Non pregnant	5.24 ± 2.81	7	5.99 ± 3.21	18.50 ± 2.61	11	6.04 ± 0.63	n.s.	n.s.
Day 7	12.91 ± 1.48	15	1.76 ± 0.679	50.25 ± 6.81	20	5.04 ± 1.48	p<0.001	n.s.
Day 21	27.00 ± 1.58	20	0.341 ± 0.255	27.64 ± 1.71	23	0.590 ± 0.245	n.s.	n.s.

Table 2. Calculated parameters of the dose response curves generated by β-adrenergic stimulation (Figure 10).

	Control			α-Methyldopa treated			p value (Emax)	p value (EC50)
	Emax ± SEM (%)	n	EC50 ± SEM (μM)	Emax ± SEM (%)	n	EC50 ± SEM (μM)		
Non pregnant	93.93 ± 1.94	22	0.099 ± 0.015	75.74 ± 3.14	23	0.482 ± 0.200	p<0.001	p<0.05
Day 7	68.24 ± 4.09	22	0.396 ± 0.162	77.27 ± 3.34	20	0.128 ± 0.022	p<0.05	n.s.
Day 21	81.14 ± 2.17	23	0.252 ± 0.038	64.77 ± 3.27	12	1.37 ± 0.265	p<0.001	p<0.001

Results of radioligand-binding experiments

In an effort to find explanation to our results received in isolated organ experiments we decided to measure the density and affinity of α_2 and β_2 adrenergic receptors using radioligand binding technique.

The measured density and affinity values of the saturation plots, are presented in Figures 11, 12, 13 and 14. As for the α_2-adrenergic receptors, both B_{max} and K_d were higher on day 7 of pregnancy than in the non-pregnant state, but both parameters had returned to the non-pregnant value at term in pregnancy. The only treatment-dependent significant change was a decrease in K_d measured on day 7 of pregnancy. No gestation-dependent change in the density of β-adrenergic receptors was detected, while K_d was increased by the end of pregnancy, and there was a treatment-dependent significant increase on Day 21.

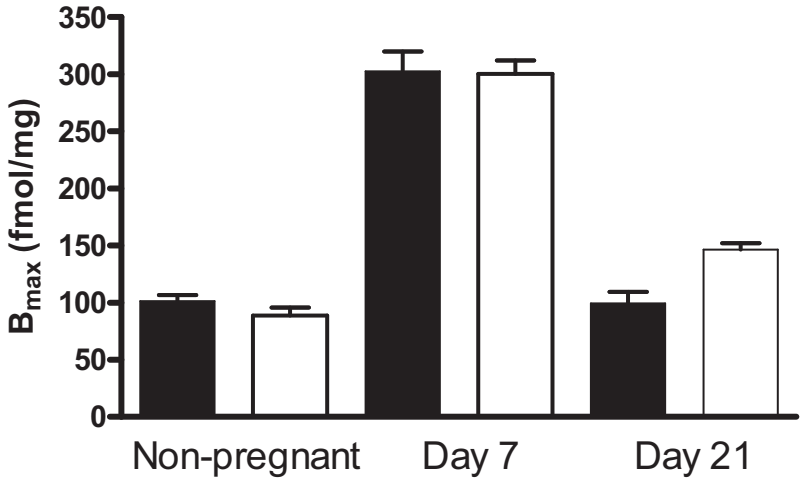

Figure 11. The measured B_{max} values of α_2-receptors ■ indicates control values, and □ indicates α-methyldopa-treated values.

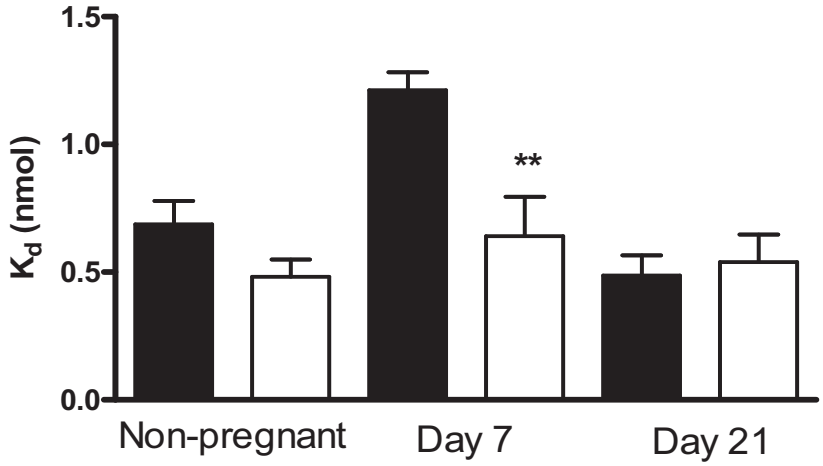

Figure 12. The measured K_d values of α_2-receptors. ■ indicates control values, and □ indicates α-methyldopa-treated values. ** denote P<0.01 as compared with the nontreated value.

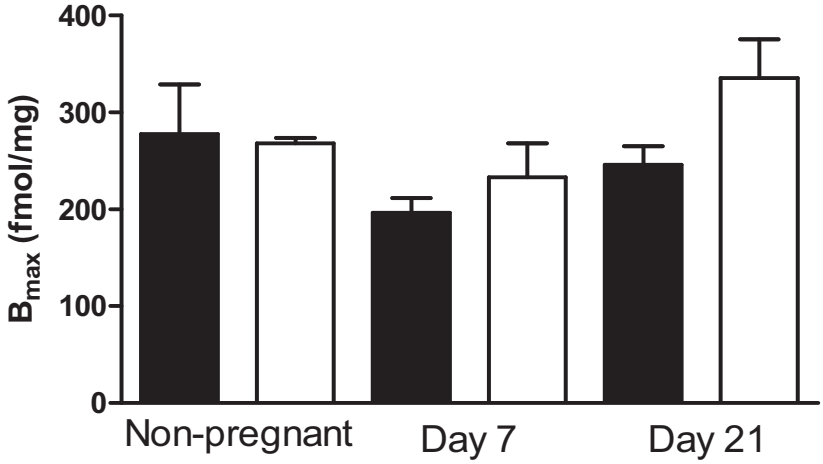

Figure 13. The measured B_{max} values of β_2-receptors. ■ indicates control values, and □ indicates α-methyldopa-treated values.

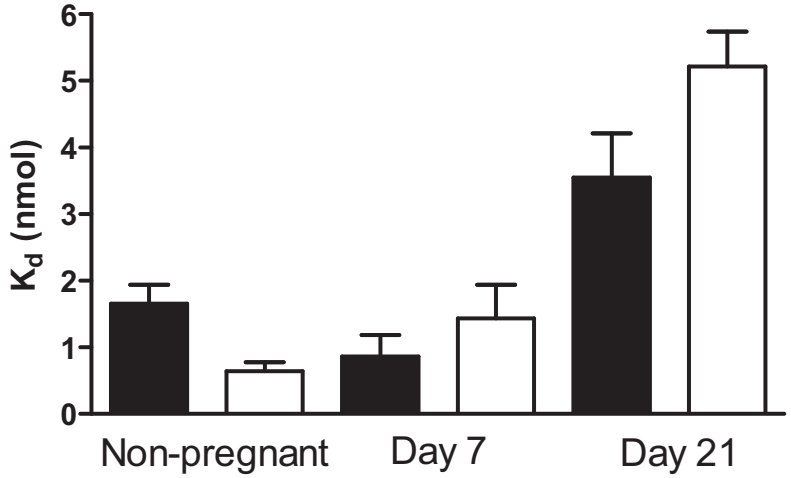

Figure 14. The measured K_d values of β_2-receptors. ■ indicates control values, and □ indicates α-methyldopa-treated values.

Discussion

Degeneration of the adrenergic nerves in the uterus during pregnancy is a well-described phenomenon in several species like guinea-pig (Bell and Malcolm, 1978), rat (Haase *et al.*, 1997) and human (Wikland *et al.*, 1984). Since this phenomenon can be found in many mammals, this suggests that pregnancy-induced adrenergic denervation might have a general importance, it can prevent the adrenergic activation of uterus during pregnancy. Adrenergic activation of uterus through α-adrenergic receptors would increase myometrial contractility and evoke vasoconstriction and these consequences may lead to pre-term delivery and would decrease blood flow of uteroplacental unit. Although in late pregnancy noradrenaline infusion administered to guinea-pigs did not affect uterine and placental flow, however vaginal and cervical blood flows were decreased (Martensson and Carter, 1982). In view of the fact that the denervated myometrial arteries become dilated and only the larger arteries retain their innervation to a minimal extent, it seems conceivable that these larger vessels take over the control of the uteroplacental resistance, permitting an elevated blood flow (Haase *et al.*, 1997). This finding is in accordance with the report of a pregnancy-induced increase in the nerve fibres in the uterine artery of the guinea-pig (Mione and Gabella, 1991). It is not clear at present how this denervational phenomenon is evoked, and whether it is believed to be restricted only to the uterus but all of the relevant data are coming from histochemical data, however this was never investigated earlier from a functional point of view. It may happen that this phenomenon is functionally more generalized. The fact that the implantation area becomes denervated first favours a causative factor of foetoplacental origin. This is in line with the unchanged innervation of the uterine-horn-devoid foetus of the late-pregnant guinea-pig (Lundberg *et al.*, 1987). Beside pregnancy there are a lot of other factors which influence innervation or denervation of the uterus. Several physiological processes like puberty and oestrus cycle have a profound effect on uterine sympathetic nerve system (Zoubina *et al.*, 1998, Zoubina and Smith, 2000, Chavez-Genaro *et al.*, 2002). It is described in many articles that oestrogen decreases the noradrenaline content of rat or guinea-pig uterus (Rydhstrom *et al.*, 1990, Brauer *et al.*, 1995). On the other hand in oestrogen receptor knockout-mice uterine hyperinnervation was reported (Zoubina and Smith, 2001).

We examined the decrease in function of the adrenergic neurons, and our functional approach is concluded to have two advantages. Firstly, we could detect a significant decrease in the

function of the myometrial adrenergic nerves as early as day 7 of pregnancy. Although histochemical examination was not a part of our present study, it was reported previously to be detectable at the end of the second third of pregnancy (Klukovits *et al.*, 2002). At the end of the first third of pregnancy, there were significant differences in noradrenaline release and uptake between the implantation and inter-implantation sites. This suggests that there are also foetoplacental factors responsible for the pregnancy-induced adrenergic denervation.

A further advantage of the approach used here is the ability to detect functional change which cannot be followed by structural investigations. This functional approach also has a disadvantage: we were not able to differentiate between muscle tissue and vessels.

During our investigation, as it was described earlier, we also measured the uptake capacity of tissues. The results of uptake capacity are presented as dpm/mg tissue without normalization for a substantially increasing myometrial weight during gestation. The distinction between areas within a uterine horn made our results inappropriate for normalization, as decreasing uptake capacity during gestation is considered to be a consequence of the degeneration of the adrenergic nerves and a dilution of the remaining fibres. On the other hand, the interpretation of the release of the transmitter – that is, the fractional release – is independent of the weight of the sample and that of the organ taken from, meaning that it reflects purely the functional deterioration of the sympathetic system.

The innervation of the cervix is reported to be unchanged in humans (Bryman *et al.*, 1987, Norstrom and Bryman, 1989) and in the guinea-pig during pregnancy (Alm *et al.*, 1979, Lundberg *et al.*, 1987). Only limited data are available on the rat. Our results clearly reveal a substantial deterioration of adrenergic functions in the cervix, disclosed by the transmitter uptake capacity, but not by the EFS-evoked release. It could be suggested, therefore, that this capacity is a more sensitive feature of the adrenergic nerve function than the transmitter release. An alternative explanation for this contradiction is that the decrease in cervical uptake capacity is solely a result of a ‘spacing’ effect due to the growth of the cervix during pregnancy. In the early postpartum period, however, both parameters indicate inhibition in the cervix. This deterioration of the cervical adrenergic function could be explained by the intensive physical stretching during delivery. Distension was suggested previously to be a factor responsible for denervation in the uterus and bladder, too (Owman *et al.*, 1980, Tammela *et al.*, 1990). It was demonstrated that the total amount of nerve growth factor and NGF mRNA are increasing in rat after delivery (Varol *et al.*, 2000). This newly synthesized

growth factor is probably responsible for the reinnervation of the myometrium and the cervix. This concept is supported by the correlation between the NGF level of a target organ and its sympathetic innervation (Korsching and Thoenen, 1983). However, this correlation is missing in the female reproductive tract of the guinea-pig and rat, indicating that NGF is not the predominant regulator of the innervation in these organs (Brauer *et al.*, 2000). The other possible key factor in gestational denervation and postpartum restoration is the receptivity of the myometrium, as evidenced by in oculo transplantation experiments. Myometrial samples from virgin guinea-pigs transplanted into the anterior eye chamber became organotypically innervated by the host superior cervical ganglion. In contrast, samples from postpartum donors were approached, but not innervated (Brauer *et al.*, 1998).

Early reinnervation is detected immunohistochemically 48 h after delivery (Haase *et al.*, 1997). However, our results indicate that the functions of the noradrenergic nerves in the myometrium and cervix have not recovered completely by postpartum weeks 4 and 2, respectively. The basic mechanism of pregnancy-induced degeneration of the adrenergic fibres in the myometrium and cervix remains enigmatic, as does the reinnervation following delivery. Our results contribute to an understanding of the phenomenon, as the functional deterioration has been shown to start earlier than the structural denervation, and the restoration requires a longer period.

α-Methyldopa, a commonly used agent in the treatment of hypertension during pregnancy (Frishman *et al.*, 2005), is considered one of the safest drugs because of the huge amount of experience relating to its clinical use (Sibai, 1996, Magee, 2001, Frishman *et al.*, 2005). Despite the large body of clinical experience, and its long use as an antihypertensive, its mechanism of action has been reconsidered several times since the recognition of its antihypertensive effect (Oates *et al.*, 1960). Earlier works suggested a peripheral site of action, involving either the inhibition of transmitter synthesis or the metabolism to α-methylnoradrenaline, which acts as a “false” transmitter, thereby decreasing the sympathetic activity (Henning and Rubenson, 1971, Day *et al.*, 1973, Nakamura *et al.*, 1980). It was later proved that α-methyldopa acts centrally, though peripheral effects were not excluded (van Zwieten *et al.*, 1984). Next, it was found that the main metabolite of α-methyldopa is a high-affinity agonist of α_2-adrenergic receptors (Schloos *et al.*, 1987). It was also suggested that the metabolites of α-methyldopa may act at almost all types of adrenergic receptors (Goldberg *et al.*, 1982). According to the most widely accepted concept, α-methyldopa exerts its

antihypertensive action through the stimulation of α_2-adrenergic receptors within the central nervous system, causing a decrease in the efferent sympathetic tone. Various antihypertensive agents are used in pregnancy beside methyldopa, like β-blockers, peripherally acting α-adrenergic antagonists, calcium channel blockers, direct vasodilators, diuretics. Although none of them are ideal for the treatment, especially ACE-inhibitors and AT II antagonists. β-blockers increase the risk of intrauterine growth restriction, may cause neonatal bradycardia, peripherally acting α-adrenergic antagonists have side effects like postural hypotension and palpitation also in non-pregnant populations therefore they are not in the first line of antihypertensive therapy. Calcium channel blockers may induce maternal hypotension and associated fetal distress. Direct vasodilators may also cause maternal hypotension and Cesarean section and low APGAR score as consequence. Diuretics are not used widely among obstetricians since they have a significant effect on plasma volume. ACE inhibitors and AT II antagonists are not used in second and third trimester since their use is associated with the following fetal problems: oligohydramnios, distress, and with the following neonatal problems: renal failure, pulmonary hypoplasia, hypocalvaria and intrauterine growth restriction (Magee, 2001). In spite of the several decades of obstetrical use of α-methyldopa, the available information on a direct uterotropic effect is very limited. Our previous experiments proved that a superfusion technique is a reliable approach for a functional investigation of the myometrium.

Our transmitter liberation results revealed that α-methyldopa all but abolished the electrically evoked noradrenaline peak in the estrus and in the early pregnant state. Implantational and inter-implantational sites of the pregnant uterus were processed separately because a large body of evidence suggests that many uterine functions can exhibit site-dependent differences. In the implantational sites of the guinea pig, the pregnancy-induced adrenergic denervation starts earlier, as evidenced by immunohistochemical studies, in good agreement with the activity of tyrosine hydroxylase in the uterine tissue (Bell and Malcolm, 1978, Alm *et al.*, 1979). These findings were reinforced by our previous observation that the implantation site corresponded to an earlier and more marked decrease in transmitter-uptake capacity and release.

In the present experiments, treatment with α-methyldopa was associated with a more marked inhibition of liberation at the inter-implantational sites, while in the case of implantational sites only the first stimulation resulted in a significantly smaller amount of liberated noradrenaline.

However, the release is similarly abolished from both α-methyldopa-treated sites on the 7th day of pregnancy, indicating that the difference in the significance of the release pattern is caused by the more marked physiological denervation at the implantational sites. Hence, it could be speculated that α-methyldopa treatment can "speed up" the physiological degeneration of the adrenergic fibers of the pregnant uterus. The results of the tissue radioactivity also favour this concept. By the time of late pregnancy this denervation has progressed, leading to the ineffectiveness of electrical stimulation, and thus the α-methyldopa treatment cannot inhibit the release. The mechanism of the transmitter release-inhibiting effect of α-methyldopa is an unanswered question, but it is in good agreement with the finding of Ihalainen and Tanila that the α_2-agonist dexmedetomidin, infused locally, decreased the liberation of noradrenaline in the nucleus accumbens of the mouse (Ihalainen and Tanila, 2004). Their study proved that this effect is mediated through the α_{2A} type of adrenergic receptors.

The superfusion technique is suitable for characterization of the presynaptic functions of the transmission, i.e. the transmitter uptake and release capacity. As far as the postsynaptic surface is concerned, a separate set of experiments was carried out on isolated uterine rings to characterize the changes elicited in the contractility responses by α- and β-adrenergic agonists (noradrenaline and terbutaline, respectively).

The contractions mediated by α-adrenergic receptors were not affected by α-methyldopa in non-pregnant and late-pregnant rats, but were significantly increased in early pregnancy. Treatment resulted in the lower efficacy and potency of terbutaline in non-pregnant and late-pregnant animals, while no change was found in the early-pregnant rats. Combining these findings, we concluded the same change for all 3 stages of gestation: the overall effects of sympathomimetics were shifted toward an increased contractility either by a decreased β-receptor-mediated relaxation or by a potentiated α-receptor-mediated contraction. The reasons for these gestational age and treatment-dependent changes in the effects of the adrenergic agonists are not understood. The higher affinity of α_2-receptors for the ligand [^{3}H]RX 821002 is in good agreement with the increased efficacy of noradrenaline in evoking contractions. However, the role of α_1-receptors in the α-methyldopa-induced higher contractility cannot be excluded. The mechanism of the decreased relaxing effect of terbutaline in virgin rats and in late pregnancy (day 21) is also a puzzle. Characteristic pregnancy-dependent changes in the results of radioligand-binding experiments were found in the case of the α_2-adrenergic

receptor. Its K_d value showed a maximum in early pregnancy, followed by a decline to its non-pregnant value. α-Methyldopa treatment decreased this maximum, which is in line with our hypothesis, i.e. the treatment can mimic a later phase of the physiological gestation-induced development of the myometrium. A large body of experimental data indicates a functional interplay between α_2- and β-adrenergic receptors (Woodcock and Johnston, 1980, Nomura *et al.*, 1984, Kitamura and Nomura, 1985, Johnston and Majewski, 1986, Nakamura *et al.*, 1991, Falkay *et al.*, 1994). The densities of these receptors change in parallel in the human brain in advancing age and in different pathological states (Sastre *et al.*, 2001). Independently of the underlying mechanism, α-methyldopa-induced alterations in the pharmacological effects of sympathomimetics can be of therapeutic importance, presuming that a similar final effect can develop in the human body. As many over-the-counter preparations contain α-receptor agonists (e.g. nasal decongestants), their usage can seriously increase the uterine contractility when the concomitant pregnancy-induced hypertension is treated with α-methyldopa. On the other hand, the decreased relaxant effect of terbutaline indicates that α-methyldopa can make β-mimetic-based tocolytic therapy less effective, thereby increasing the danger of premature delivery.

As there are no direct data that exclude modulation of the effects of adrenergic agonists similar to our finding, sympathomimetics should be used with great care in pregnant women treated with α-methyldopa.

The basic mechanism of pregnancy-induced degeneration of the adrenergic fibres in the myometrium and cervix remains enigmatic, as does the reinnervation following delivery. Our results contribute to an understanding of the phenomenon, as the functional deterioration has been shown to start earlier than the structural denervation, and the restoration requires a long period. The outcome of our experiments leads us to conclude that superfusion can be utilized as a model system for investigation of the effects of pharmacological manipulation and pathological states (for example, pregnancy-induced hypertension or gestational diabetes) on the denervation procedure.

Based on our investigations it can be concluded that superfusion technique is suitable to examine myometrial adrenergic transmission from a functional point of view. We were able to detect significant differences in transmitter uptake and in stimulation evoked transmitter release during gestation and as a consequence of pharmacological intervention.

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Appendix

Copies of Scientific publications related to the subjects of the thesis

REPRODUCTION
RESEARCH

A rat model for functional characterization of pregnancy-induced denervation and postpartum reinnervation in the myometrium and cervix: a superfusion study

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Abstract

The pregnancy-induced rapid degeneration of the adrenergic nerves innervating the uterus is a well-known but poorly understood phenomenon. Since most of the published investigations were carried out by histological assay, our aim was to describe the loss of the adrenergic function during pregnancy and the reinnervational procedure in the postpartum period. Myometrial and cervical samples from rats were loaded with [^{3}H]noradrenaline and then transferred into a chamber for superfusion. After a wash-out period, fractions were collected. The fifth and fifteenth fraction tissues were stimulated with an electric field. The [^{3}H]noradrenaline contents of the fractions were determined, together with the amount remaining in the tissue. The myometrial [^{3}H]noradrenaline release was substantially decreased in early pregnancy, and absent in the late stage. Differences in release profile were detected between the implantation sites and the interimplantation areas. As a refinement of the results of previous histochemical studies, the noradrenergic functions of the cervix were found to be deeply affected in the early postpartum period. The pregnancy-induced denervational procedure can be followed by means of a superfusional technique after [^{3}H]noradrenaline loading. As our technique is considered to be similar in sensitivity to histological methods, superfusion can be regarded as a model for functional investigations of pregnancy-induced denervation.

Reproduction (2005) **130** 743–749

Introduction

Pregnancy induces dramatic changes in practically all of the physiological functions in mammals, including hormonal environment and metabolic status, but the most impressive changes are restricted to the reproductive organs. Beside the several-fold increase in the mass of the myometrial smooth muscle, the uterus undergoes a well-characterized but little understood remodelling of its innervation. The uterus of the non-pregnant rat is supplied by adrenergic (Adham & Schenk 1969), cholinergic (Bell 1972) and peptidergic fibres (Papka *et al.* 1985), all of them innervating both the vasculature of the myometrium and the smooth muscle itself. This remodelling involves a profound denervation procedure, in which the histologically visualized density of these nerves decreases substantially as the gestation continues, and a reinnervational process starts after delivery (Haase *et al.* 1997). The degeneration of the nerves was initially presumed to be restricted to the adrenergic system (Thorbert *et al.* 1979), but a large body of evidence now indicates that all of the nerves in the uterus, including the cholinergic and peptidergic fibres, undergo this denervation during gestation (Hervonen *et al.* 1973, Moustafa 1988, Haase *et al.* 1997). The decreased amounts of functional storage vesicles in the uteri of guinea-pigs at term pregnancy are consistent with the proposed pregnancy-induced degeneration of adrenergic nerves (Fried *et al.* 1985). As the adrenergic system plays a crucial role in the regulation of the contractility of the pregnant uterus, the degeneration of the adrenergic nerves of the uterus is concluded to be most important, and this phenomenon is subjected to the most intensive investigation. Ultrastructural characterization of pregnancy-induced adrenergic denervation is frequent, with most studies applying immunohistochemical methods. These techniques are suitable for a detailed description of the progression of the denervational procedure and for differentiating between the innervation of the smooth muscle and that of the vessels supplying the myometrium. It is conceivable, however, that there could be some functional change in the uterine innervation that can be detected before signs of degeneration of the nerve. Investigation of the pharmacological reactivity and a description of the adrenergic receptor status as a function of the gestation

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could be regarded as a functional approach to myometrial denervation (Engstrom *et al.* 1997). When these methods are used, however, the final conclusion is influenced by numerous other factors which are also reported to change during pregnancy; for example, the density and affinity of the targeted receptor, and its coupling to the signal mechanism (Legrand *et al.* 1993, Mhaouty *et al.* 1995). Accordingly, the aim of our present work was to describe an animal model that is suitable for characterizing the adrenergic denervation of the pregnant uterus, and the reinnervation in the postpartum period, from a purely functional aspect. We hypothesized that deterioration or loss of the function of the adrenergic fibres occurs earlier than the structural changes during pregnancy, and the function recovers later than the structure after delivery. As the presynaptic uptake and stimulated release of the transmitter can be regarded as a functional marker of the adrenergic nerves, a superfusion technique was chosen. With this method, the electrically evoked release of [^{3}H]noradrenaline was investigated as a function of gestational age and in the early postpartum period. As the histological data suggest that the cervical adrenergic innervation remains intact or little affected, whereas only limited data are available on its functional changes during pregnancy, cervical samples were additionally investigated by the same method (Bryman *et al.* 1987, Lundberg *et al.* 1987, Nostrom & Bryman 1989).

Materials and Methods

Sprague–Dawley rats (200–250 g for females) were mated in a special cage in the early morning; copulation was determined by the presence of a copulation plug or sperm in a native vaginal smear. The day of conception was considered to be the first day of pregnancy.

Release of [^{3}H]noradrenaline

Pregnant and non-pregnant female Sprague–Dawley rats were killed by cervical dislocation. Samples of uterine and cervical tissue (20–30 mg) were dissected; the samples from the implantation and interimplantation sites were processed separately. Myometrial samples were cleared from connective tissue and endometrium. The wet weights of the samples were measured, and they were minced and incubated with 10^{-7} M [^{3}H]noradrenaline at 37°C for 60 min. The samples were then washed three times with de Jongh buffer, and the pieces were placed into superfusion chambers (Experimetria, Budapest, Hungary), which were superfused continuously for 60 min at a flow rate of 1 ml/min with de Jongh buffer containing the monoamine oxidase (MAO) inhibitor pargyline, the noradrenaline-reuptake inhibitor desipramine and the extraneuronal reuptake inhibitor deoxycorticosterone (each 10 mM). The composition of the buffer was 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4 and 6 mM glucose, pH 7.4. The solution was maintained at 37°C and equilibrated throughout the experiment with O_2 containing 5% (v/v) CO_2. After a 60-min wash-out period, a total of 22 3-min fractions were collected. At the end of the experiment, the tissue samples were solubilized in 1 ml Solvable (Canberra-Packard, Budapest, Hungary) for 3 h at 60°C. The ^{3}H content in each 3-min fraction and tissue solution was determined with a liquid scintillation spectrometer.

Electrical field stimulation (EFS) consisting of square-wave pulses was applied to the tissues, using a programmable stimulator (Experimetria). EFS was applied twice after the wash-out period, during fractions 5 and 15. Each period of stimulation consisted of 360 pulses (voltage, 40 V; pulse width, 2 ms; frequency, 2 Hz; these parameters are suitable for neural stimulation).

The [^{3}H]noradrenaline contents in the fractions were expressed as fractional release. This is the amount of labelled transmitter liberated during a 3-min fraction as a percentage of the actual radioactivity content in the tissue at the time of sampling. Peak releases were calculated by subtraction of the radioactivity of the fourth and fourteenth fractions from that of the fifth and fifteenth fractions, respectively. All experimental animal protocols satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the University of Szeged.

Drugs

Pargyline, desipramine and deoxycorticosterone were from Sigma-Aldrich (Budapest, Hungary). (−)-7-[^{3}H](*N*)-Noradrenaline hydrochloride (specific activity, 7.94 Ci/mmol) was from Perkin Elmer Life Sciences (Boston, MA, USA).

Statistical analysis

Differences between mean values were evaluated by using one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. Differences between implantation and interimplantation sites were evaluated by using the unpaired *t* test. Statistical analysis of the data was performed with GraphPad Prism 2.01 (Graph Pad Software, San Diego, CA, USA). All reported data are mean results from at least six independent experiments.

Results

Results of tissue activity determination

The tissue activity (expressed in d.p.m./mg tissue) was used to describe the uptake capacity of the sample for [^{3}H]noradrenaline. This was highest in the non-pregnant state and a gradual decrease was seen during pregnancy (Fig. 1). On day 7, tissue uptake of the labelled transmitter for the implantation sample was significantly less than that for the interimplantation myometrium. A similar trend was experienced in the cervix, resulting in a significantly lower tissue activity from day 14. As far as the postpartum period was

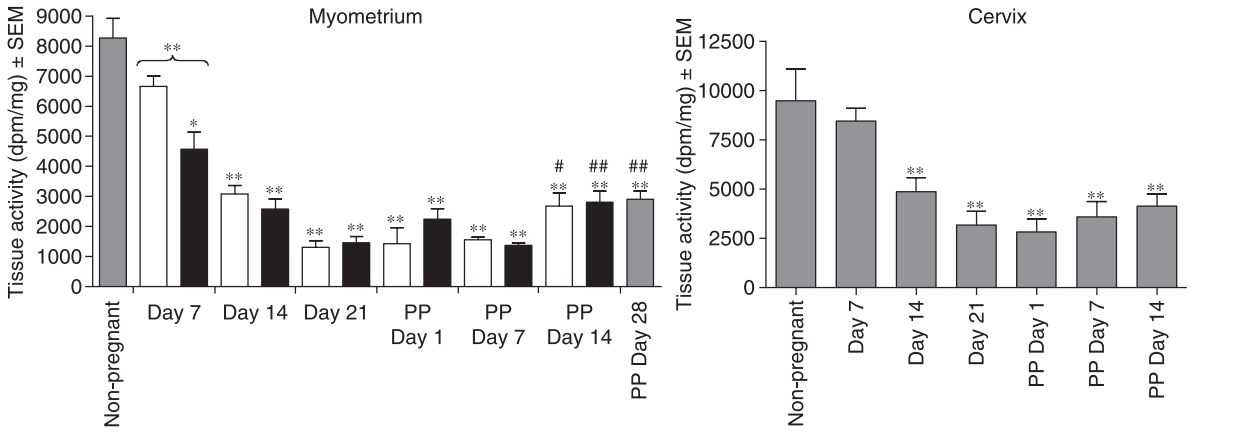

Figure 1 [^{3}H]Noradrenaline-uptake capacity of myometrial and cervical samples during gestation and the postpartum (PP) period. * and ** denote $P < 0.05$ and $P < 0.01$ as compared with the non-pregnant value, respectively; # and ## denote $P < 0.05$ and $P < 0.01$ as compared with the day-21 values, respectively. Black bars, implantation sites; white columns, interimplantation sites (distinction between the two sites is not possible 21 days after delivery).

concerned, tissue activity of the uterus at both the implantation and interimplantation sites and in the cervix remained lower than before pregnancy throughout the 28 days of the investigation. In contrast with the period of pregnancy, no site-dependent difference was observed in the myometrium. However, when the postpartum results were compared with the day-21 results, the myometrial samples displayed significantly higher values after 14 days, which points to a slow but detectable reinnervation procedure. The tissue activities of the postpartum cervical samples were not higher than the day 21 value.

Results of stimulated [^{3}H]noradrenaline release

EFS evoked a substantial [^{3}H]noradrenaline release in both the uterus and the cervix isolated from non-pregnant rats (Figs 2 and 3). Two EFSs were applied to obtain information on the release capacity of the tested tissue. The second stimulus resulted in a smaller transmitter peak than the first. In the myometrial tissue, a gradual decrease was detected in the peak evoked by EFS during the gestation. The amount of [^{3}H]transmitter released became less and less as the pregnancy progressed, and a gradual increase could be measured during the first 28 days of the postpartum period (Fig. 4). In general, it was found that the peak elicited by the second EFS decreased in a more sensitive way than did the first one; that is, as compared with the non-pregnant results a significant difference ($P < 0.01$) could be detected earlier: day 7 versus day 21 for the second and first peaks, respectively (Fig. 2). A substantial and significant difference was found between the implantation and interimplantation sites of the uterus in the early pregnant state, indicating that the loss of the adrenergic nerve function starts in the implantation area. As concerns the cervical samples, a gradual tendency of the EFS-evoked [^{3}H]noradrenaline release to decrease was observed during pregnancy, but these changes were not significant statistically (Fig. 3).

In the postpartum period, a gradual increase in the function of the noradrenergic nerves could be demonstrated in the uterus, but even after 28 days the EFS-induced release was reduced and the stimulated release of [^{3}H]noradrenaline was not significantly different from that on the last day of pregnancy. In contrast with the denervational process, reinnervation could be detected only in the first peak. It is assumed that the first EFS exhausted the limited transmitter capacity of the regenerating nerves, resulting in a decreased second peak. The EFS-evoked release of [^{3}H]noradrenaline from the cervical samples was significantly suppressed in the early postpartum period, and approximated the non-pregnant level 14 days after delivery (Fig. 3). Cervical adrenergic reinnervation was followed only up to postpartum day 14.

Discussion

Degeneration of the adrenergic nerves in the uterus during pregnancy is a well-described phenomenon in the guinea-pig (Bell & Malcolm 1978), rat (Haase *et al.* 1997) and human (Wikland *et al.* 1984). Its common occurrence in mammals suggests that this kind of uterine denervation is of physiological importance, contributing to the quiescence of the uterus during pregnancy. Since activation of the sympathetic system would result in increases in myometrial contractility and vasoconstriction, the loss of the adrenergic fibres may prevent all of these potentially harmful consequences (Owman 1981). In view of the fact that the denervated myometrial arteries become dilated and only the larger arteries retain their innervation, it seems conceivable that these larger vessels take over the control of the uteroplacental resistance, permitting an elevated blood flow (Haase *et al.* 1997). This finding is in accordance with the report of a pregnancy-induced increase in the nerve fibres in the uterine artery of the guinea-pig (Mione & Gabella 1991).

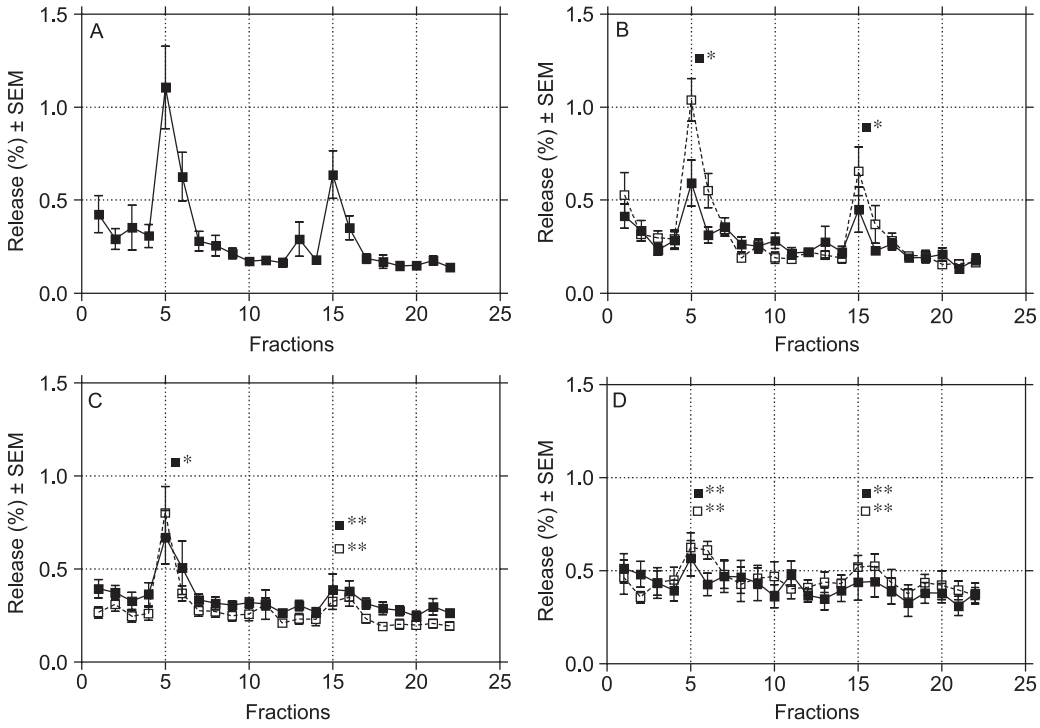

Figure 2 EFS-evoked fractional [^{3}H]noradrenaline release from myometrial samples at oestrus (A), and on days 7 (B), 14 (C) and 21 (D) of pregnancy. * and ** denote $P < 0.05$ and $P < 0.01$ as compared with the non-pregnant value, respectively. During gestation, ■ and □ indicate release from the implantation and interimplantation sites, respectively.

It is not clear at present how this denervational phenomenon is evoked, and whether it is generalized or not. The fact that the implantation area becomes denervated first favours a causative factor of foetoplacental origin. This is in line with the unchanged innervation of the uterine-horn-devoid foetus of the late-pregnant guinea-pig (Lundberg *et al.* 1987). However, a large body of evidence indicates that the innervation of the uterus demonstrates pregnancy-independent plasticity, and physiological factors such as puberty, the oestrus cycle and sexual steroid manipulations can result in changes – either increases or decreases – in the nerve pattern (Van Orden *et al.* 1980, Juorio *et al.* 1989, Brauer *et al.* 1992, Zuobina & Smith 2001). Uterine hyperinnervation has also been reported in oestrogen receptor-knockout mice, which is a further argument in favour of a generalized mechanism responsible for denervation (Zuobina & Smith 2001). As the basic reason for the phenomenon is unknown, the time of its initiation is also incompletely defined.

We examined the decrease in function of the adrenergic neurones, a functional approach that is concluded to have two advantages. Firstly, we could detect a significant decrease in the function of the myometrial adrenergic nerves as early as day 7 of pregnancy. Although histochemical examination was not a part of our present study, it was reported previously to be detectable at the end of the second third of pregnancy (Klukovits *et al.* 2002). At the end of the first third of pregnancy, there were significant differences in noradrenaline release and uptake between the implantation and interimplantation sites. This suggests that there are also foetoplacental factors responsible for the pregnancy-induced adrenergic denervation. The results of uptake capacity are presented as d.p.m./mg tissue without normalization for a substantially increasing myometrial weight during gestation. The distinction between areas within a uterine horn made our results inappropriate for normalization, as decreasing uptake capacity during gestation is considered to be a consequence of the degeneration of the adrenergic nerves and a dilution of the remaining fibres. On the other hand, the interpretation of the release of the transmitter – that is, the fractional release – is independent of the weight of the sample and that of the organ taken, meaning that it reflects purely the functional deterioration of the sympathetic system.

A further advantage of the approach used here is the ability to detect functional change which cannot be followed by structural investigations. The innervation of the cervix is reported to be unchanged in humans (Bryman *et al.* 1987, Nostrom & Bryman 1989) and in the guinea-pig during pregnancy (Alm *et al.* 1979, Lundberg *et al.* 1987). Only limited data are available on the rat. Our results clearly reveal a substantial functional deterioration in the cervix, disclosed by the transmitter uptake capacity, but not by the EFS-evoked release. It could be suggested, therefore, that this capacity is a more sensitive feature of

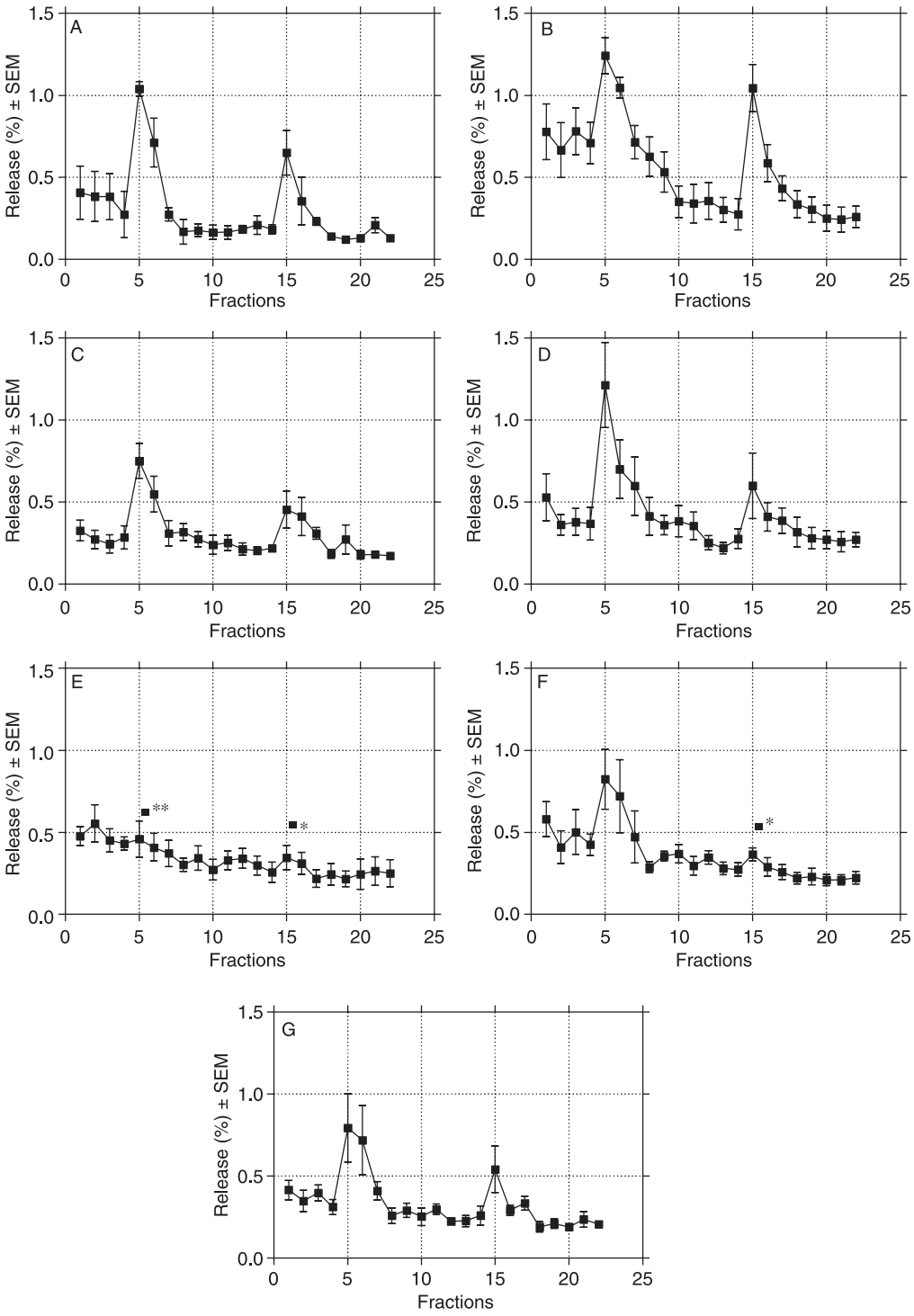

Figure 3 EFS-evoked fractional [^{3}H]noradrenaline release from cervical samples at oestrus (A), on days 7 (B), 14 (C) and 21 (D) of pregnancy, and on postpartum days 1 (E), 7 (F) and 14 (G). * and ** denote $P < 0.05$ and $P < 0.01$ as compared with the non-pregnant value, respectively.

the adrenergic nerve function than the transmitter release. An alternative explanation for this contradiction is that the decrease in cervical uptake capacity is solely a result of a 'spacing' effect due to the growth of the cervix during pregnancy. In the early postpartum period, however, both parameters indicate inhibition in the cervix. This deterioration of the cervical adrenergic function could be explained by the intensive physical stretching during

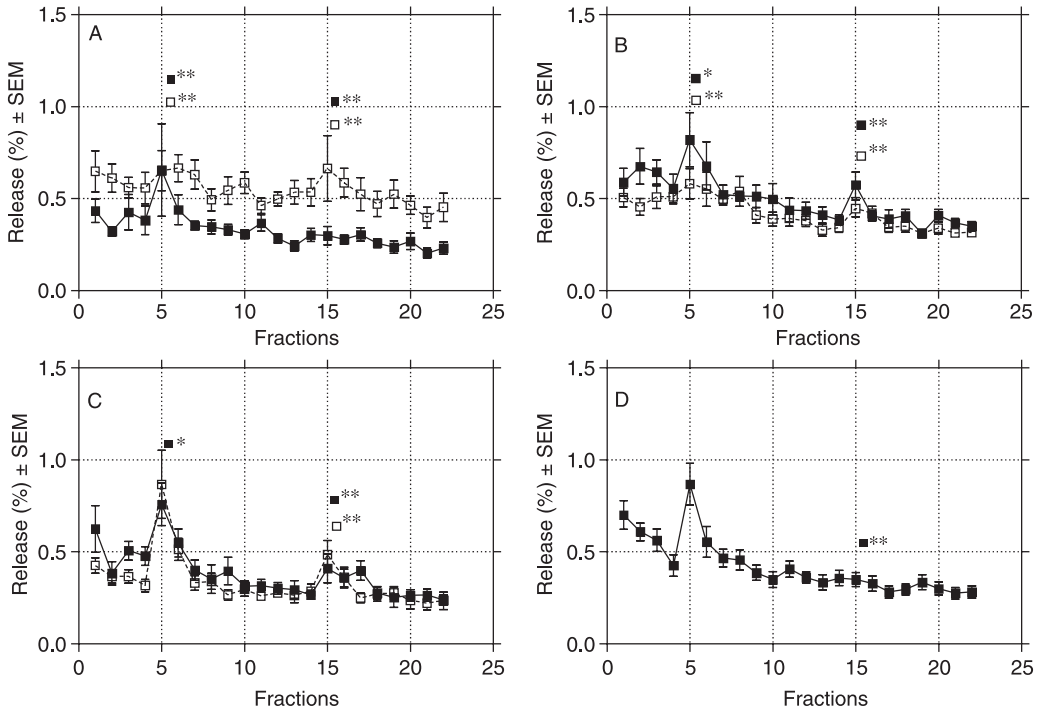

Figure 4 EFS-evoked fractional [^{3}H]noradrenaline release from postpartum myometrial samples on days 1 (A), 7 (B), 14 (C) and 28 (D). * and ** denote $P < 0.05$ and $P < 0.01$ as compared with the non-pregnant value, respectively. In A–C, ■ and □ indicate release from the implantation and interimplantation sites, respectively (distinction between the two sites is not possible 21 days after delivery).

delivery. Distension was suggested previously to be a factor responsible for denervation in the uterus and bladder too (Owman *et al.* 1980, Tammela *et al.* 1990).

It was demonstrated that the total amount of nerve growth factor (NGF) and its mRNA are increased substantially by late pregnancy and return to the mature virgo level by 7 days after delivery (Varol *et al.* 2000). This newly synthesized growth factor is probably responsible for the reinnervation of the myometrium and the cervix. This concept is supported by the correlation between the NGF level of a target organ and its sympathetic innervation (Korsching & Thoenen 1983). However, this correlation is missing in the female reproductive tract of the guinea-pig and rat, indicating that NGF is not the predominant regulator of the innervation in these organs (Brauer *et al.* 2000). The other possible key factor in gestational denervation and postpartum restoration is the receptivity of the myometrium, as evidenced by in oculo transplantation experiments (Brauer *et al.* 1998). Myometrial samples from virgin guinea-pigs transplanted into the anterior eye chamber were organotypically innervated by the host superior cervical ganglion. In contrast, samples from postpartum donors were approached, but not innervated.

Early reinnervation is detected immunohistochemically 48 h after delivery (Haase *et al.* 1997). However, our results indicate that the functions of the noradrenergic nerves in the myometrium and cervix have not recovered completely by postpartum weeks 4 and 2, respectively.

The basic mechanism of pregnancy-induced degeneration of the adrenergic fibres in the myometrium and cervix remains enigmatic, as does the reinnervation following delivery. Our results contribute to an understanding of the phenomenon, as the functional deterioration has been shown to start earlier than the structural denervation, and the restoration requires a long period. Our results lead us to conclude that superfusion can be utilized as a model system for investigation of the effects of pharmacological manipulation and pathological states (for example, pregnancy-induced hypertension or gestational diabetes) on the denervation procedure.

Acknowledgements

The author's declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. I Zupkó is grateful for support from János Bolyai Research Fellowship.

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ORIGINAL ARTICLE

The effects of α-methyldopa on myometrial noradrenaline release and myometrial contractility in rat

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Abstract

Background. α-Methyldopa is a classic antihypertensive agent, used routinely in the treatment of pregnancy-induced hypertension. However, only a few data are available about its direct uterotropic effect. Accordingly, the aim of the present study was to investigate the direct effects of α-methyldopa on the myometrial adrenergic functions in rat. *Methods.* The effects of α-methyldopa on the sympathetic transmission in the non-pregnant, early pregnant and late-pregnant myometrium were investigated by a superfusion technique. Myometrial samples from control and α-methyldopa-treated (200 mg/kg i.p. for 7 days) non-pregnant, 7-day and 21-day pregnant rats were saturated with [^{3}H]noradrenaline, and the liberation evoked by electric field stimulation was determined. The contractility responses to α- and β-adrenergic stimulation were additionally characterised by generating concentration–response curves of myometrial rings to noradrenaline and terbutaline in the same arrangement. The changes in the density and affinity of the adrenergic receptors (α_2 and β_2) were investigated by a radioligand binding technique. *Results.* The treatment with α-methyldopa substantially decreased both the [^{3}H]noradrenaline uptake and release in both the non-pregnant and early pregnant uterus, while treatment-dependent changes were observed at term only in the uptake capacity. The contractility response to exogenous α-sympathomimetics was higher in the group treated in early pregnancy, and a decreased terbutaline-induced relaxation was observed in the non-pregnant state and at term. The treatment resulted in increased affinity for α_2 receptors in early pregnancy, while K_d for β_2 was increased at term. *Conclusions.* Our experimental data suggest that besides its antihypertensive effect, α-methyldopa may influence the adrenergic transmission of the pregnant uterus. Our results indicate that the agent decreases the efficacy of β_2-adrenergic agonists at term pregnancy and increases the response to α-sympathomimetics in early pregnancy.

Key words: *α-Methyldopa, myometrium, rat, denervation*

Introduction

Hypertensive disorders are the most significant complications of pregnancy, affecting approximately 10% of all pregnancies and contributing greatly to maternal and perinatal morbidity and mortality throughout the world (1). As α-methyldopa has an advantageous safety and efficacy profile, it is the most commonly used antihypertensive agent in pre-eclamptic patients, gestational hypertension and chronic hypertension in pregnancy (2–5).

α-Methyldopa is a centrally acting antihypertensive drug, which enters the biosynthesis of catecholamines (6–8), and is transformed into α-methylnoradrenaline, a potent α_2-adrenergic agonist (9). Thereby, α-methyldopa decreases the sympathetic activity of the autonomic nervous system. Its metabolites bind mainly to α_2-adrenergic receptors, but have affinity for β_2 and α_1-adrenergic receptors (9).

The uterus of both the non-pregnant and pregnant rat is supplied by adrenergic (10), as well as cholinergic (11) and peptidergic fibers (12), all innervating both the vasculature of the myometrium and the smooth muscle itself. Since catecholamines, besides oxytocin and prostaglandins, play an important role

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in the regulation of myometrial contractility during gestation (13), it seemed worthwhile investigating the direct myotropic effect of α-methyldopa as a function of gestational age.

As the information on the myometrial action of α-methyldopa is limited, we decided to investigate its effects on myometrial noradrenaline release and contractility. To determine the influence of α-methyldopa on myometrial noradrenaline release and tissue uptake, we chose a superfusion technique. In a previous study, this superfusion technique proved useful for characterisation of the pregnancy-induced adrenergic denervation by assessing the [^{3}H]noradrenaline uptake into myometrial samples, and its electrically provoked release (14). In order to examine the effect of α-methyldopa treatment on myometrial contractility, we performed isolated organ experiments. A radioligand binding technique was used to assess whether the changes in myometrial contractility caused by α-methyldopa treatment were consequences of receptorial mechanisms or whether other signal transductional pathways may be responsible for possible alterations.

Materials and methods

Female Sprague-Dawley rats (200–250 g) were mated in a special cage in the early morning; copulation was determined by the presence of a copulation plug or sperm in a native vaginal smear. The day of conception was considered the first day of pregnancy. All experimental animal protocols satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the University of Szeged.

The animals in the active group were treated with α-methyldopa (200 mg/kg, i.p.) daily for 7 days, and the experiment was performed on the 7th or 21st day. Control animals were treated with physiological saline.

Release of [^{3}H]noradrenaline

Pregnant and non-pregnant rats were sacrificed by cervical dislocation. Samples of uterine tissue (20–30 mg) were dissected; the samples from the implantation and interimplantation sites were processed separately. Myometrial samples were cleared from connective tissue and endometrium. The wet weights of the samples were measured, minced and incubated with 10^{-7} M [^{3}H]noradrenaline at 37°C for 60 min. The samples were then washed 3 times with de Jongh buffer containing the monoamine oxidase inhibitor, pargyline, the noradrenaline reuptake inhibitor, desipramine, and the extraneuronal reuptake inhibitor, deoxycorticosterone (each 10 μM). The composition of the buffer was 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4 and 6 mM glucose, pH 7.4. The solution was maintained at 37°C and equilibrated throughout the experiment with O_2 containing 5% (v/v) CO_2. After a 60-min washout period, a total of 22 3-min fractions were collected. At the end of the experiment, the tissue samples were solubilised in 1 ml Solvable (Canberra-Packard, Budapest, Hungary) for 3 h at 60°C. The ^{3}[H] content in each 3-min fraction and tissue solution was determined with a liquid scintillation spectrometer.

Electric field stimulation (EFS) was applied to the tissues during fractions 5 and 15, using a programmable stimulator (Experimetria Ltd., Budapest, Hungary). Each period of EFS consisted of 360 pulses (voltage: 40 V; pulse width: 2 ms; frequency: 2 Hz; these parameters are suitable for neuronal stimulation).

The [^{3}H]noradrenaline contents in the fractions were expressed as fractional release. This is the amount of labeled transmitter liberated during a 3-min fraction as a percentage of the actual radioactivity content in the tissue at the time of the sampling. Peak releases were calculated by subtraction of the radioactivity of fractions 4 and 14 from that of fractions 5 and 15, respectively. The total amount of isotope taken up by the tissues was also determined and expressed as dpm/mg tissue.

Isolated tissue studies

Uterine rings were taken from the uterine horns of pregnant or non-pregnant, treated or non-treated rats. Two muscle rings were sliced from both horns of the uterus and mounted vertically in a tissue bath containing 10 ml de Jongh buffer. The temperature of the tissue bath was set and maintained at 37°C, O_2 containing 5% (v/v) CO_2 was perfused continuously through the bath, and buffer was changed every 15 min. Tissue samples were equilibrated under these conditions for 90 min before the experiments were started. The initial tension of the uterus rings was set to 1.5 g, which dropped to approximately 0.5 g by the end of the equilibration period. The tensions of the myometrial rings were measured with a strain gauge transducer (SG-02, Experimetria Ltd.) and recorded with an Isosys Data Acquisition System (Experimetria Ltd.). The areas under the curves were analysed for a 5-min period after each administration of the tested substances.

Cumulative concentration–response curves were constructed for noradrenaline in the concentration

range 10^{-10} to 10^{-5} M (total: 11 concentrations). The chamber contained propranolol (10^{-5} M) to block the β-adrenergic receptors. At the end of the experiment, KCl (70 mM) was added to the chamber, and the evoked contractions were recorded for 5 min. The contractions induced by noradrenaline were expressed as a percentage of the KCl evoked contractions.

To characterise the effects of α-methyldopa on the β-adrenergic receptor-mediated myometrial relaxation, cumulative concentration–response curves were additionally constructed for terbutaline in the concentration range 10^{-8} to 10^{-5} M (total: 7 concentrations). KCl (50 mM) was added to the chamber before the start of the experiment in order to elicit an initial tension of the uterine rings which was regarded as 100% of the motor activity.

A sigmoidal curve was fitted individually to all concentration–response curves (both noradrenaline and terbutaline) and the maximal effect (E_{max}) and EC_{50} values were calculated by means of Graphpad Prism 2.01 (Graph Pad Software, San Diego, CA, USA).

Radioligand-binding studies

Membrane preparation. Uterine tissues were dissected carefully and the embryos were rapidly removed. All subsequent steps were performed at 4°C. Uteri were homogenised in 6–10 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose with an Ultra Turrax 25 homogeniser (IKA Labortechnik, Staufen, Germany). The homogenate was centrifuged at 20,000 × *g* for 10 min and the pellet was recentrifuged. The supernatants were collected and centrifuged at 50,000 × *g* for 60 min, and the pellet was used for saturation experiments. The membrane preparation was stored at −70°C until assayed. The protein concentration of the membrane fraction was measured by the method of Bradford, with bovine serum albumin as standard (15).

Saturation binding experiments

Saturation binding experiments were performed by incubating the cell membrane fraction with a range of concentrations of [^{3}H]RX 821002 (0.2–8.0 nM, α_2-adrenoceptors) and [^{3}H]ICI 118,551 (0.5–10 nM, β_2-adrenoceptors) at 25°C for 45 min. At the end of the incubation, the bound radioligand was separated from the residual free radioligand by rapid filtration on a Brandell cell harvester through Whatman GF/C filters and washed with 3 × 10 ml of ice-cold buffer (Tris–HCl, pH = 7.41). The bound radioactivity was determined in a Wallac 1409 liquid scintillation counter. The observed total binding consists of specific binding to the receptor itself, plus non-specific binding to non-receptor sites. The non-specific binding was measured with 10 μM unlabeled yohimbine and alprenolol. The specific binding was calculated as the difference between the total and the non-specific binding, and was plotted as a function of the free radioligand concentration. The equilibrium dissociation constant (K_d) and the binding capacity (B_{max}) values were calculated with GraphPad Prism software.

Data analysis of radioligand-binding studies

All assays were carried out at least 3 times in duplicate, and values are given as means ± SEM. B_{max} and K_d values of [^{3}H]RX 821002 and [^{3}H]ICI 118,551 were calculated according to Rosenthal (16). The data from the radioligand-binding experiments were analysed using the GraphPad Prism 2.01 software, utilising a non-linear regression analysis.

Drugs

Pargyline, desipramine, deoxycorticosterone, noradrenaline, terbutaline, propranolol, yohimbine and alprenolol were purchased from Sigma-Aldrich (Budapest, Hungary). (–)-7-[^{3}H](N)-Noradrenaline hydrochloride (specific activity, 7.94 Ci/mmol) was from Perkin Elmer Life Sciences (Boston, MA, USA), [^{3}H]RX 821002 (specific activity, 50 Ci/mmol) from Amersham Bioscience (UK), and [^{3}H]ICI 118,551 (specific activity, 18.8 Ci/mmol) from Tocris (UK).

Statistical analysis

Data from the superfusion experiments were evaluated by one-way ANOVA followed by Newman–Keuls post test. To calculate the differences between mean values of the EC_{50} and E_{max} of the isolated organ experiments, we have used two-way ANOVA followed by Bonferroni post test. Differences between mean values of the radioligand-binding results were evaluated by one-way ANOVA followed by Newman–Keuls post test. Statistical analyses of the data were performed with Graphpad Prism 2.01. All reported data are mean results from at least 6 independent experiments.

Results

Results of stimulated [^{3}H]noradrenaline release

EFS evoked a substantial [^{3}H]noradrenaline release in the uterus isolated from non-pregnant rats

(Figure 1). The second stimulus resulted in a smaller transmitter peak than the first stimulus. Two periods of EFS were used in order to obtain information on the release capacity of the tested tissue. For the α-methyldopa-treated non-pregnant rats, this EFS-evoked [^{3}H]noradrenaline release was almost completely abolished. On day 7 of pregnancy, the [^{3}H]noradrenaline release was decreased at both

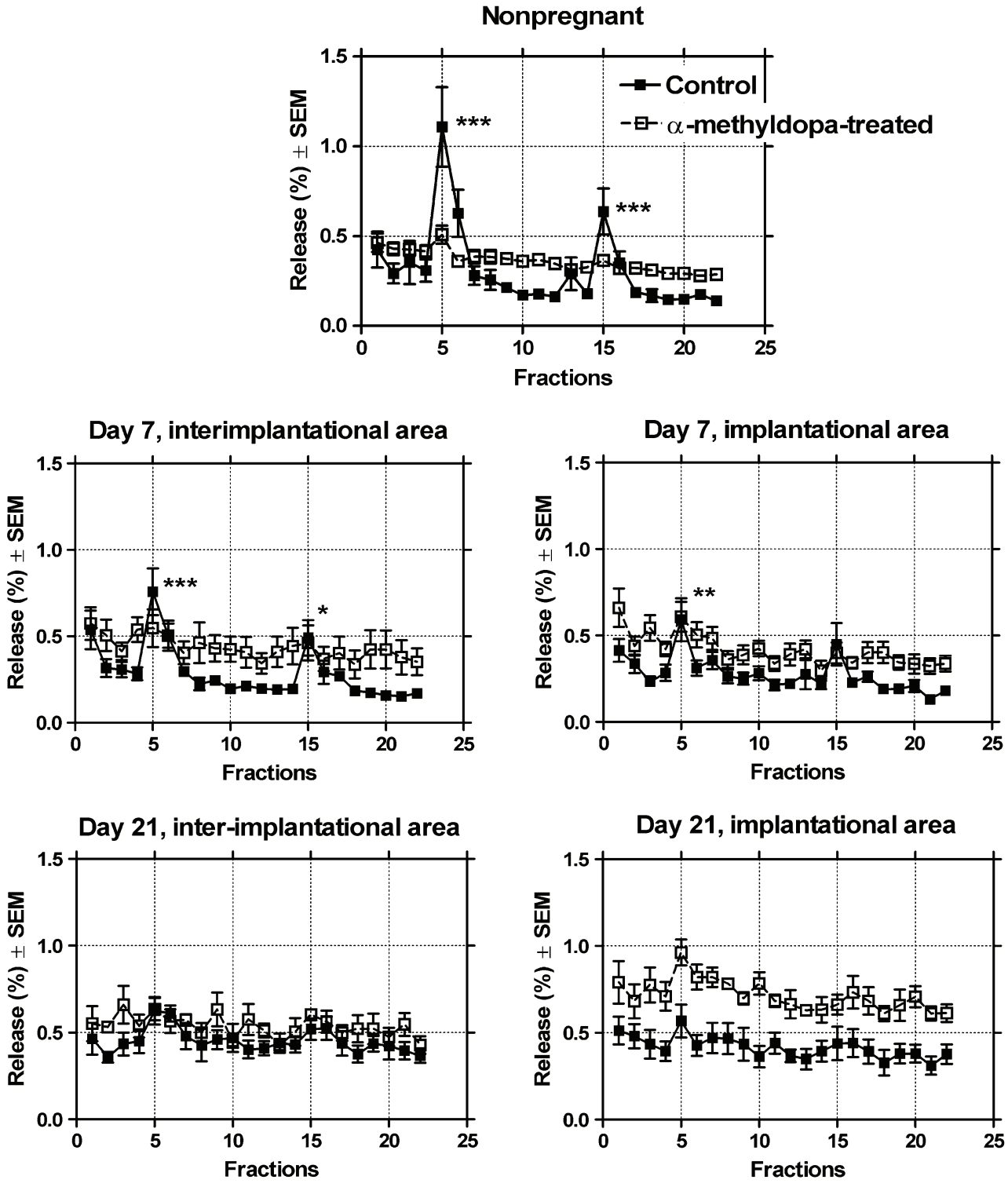


Figure 1. The effects of α-methyldopa treatment on the EFS-evoked fractional [^{3}H]noradrenaline release from myometrial samples at estrus (upper panel), on day 7 (middle panels) and on day 21 (lower panels). *p <0.05, **p <0.01 and ***p <0.001 compared with the non-treated value. ■ indicates control values, and □ indicates α-methyldopa-treated values.

implantation and interimplantation sites due to the pregnancy-induced adrenergic denervation. α-Methyldopa treatment substantially decreased the first EFS-evoked [^{3}H]noradrenaline release at both sites, while the differences of the second peaks proved significant only at the interimplantational sites. At the end of pregnancy (day 21), the amount of [^{3}H]noradrenaline released from the α-methyldopa-treated animals was unchanged compared with the control level. The elevation of the baseline release at the implantation site is presumed to be an artifact due to the manner of calculation of the fractional release: when the [^{3}H]noradrenaline release from the treated tissues was strongly decreased, the background activity became substantial, resulting in a relatively high value during a 3-min period.

Results of tissue radioactivity determination

The tissue radioactivity (expressed in dpm/mg) was used to describe the uptake capacity of the sample for [^{3}H]noradrenaline (Figure 2). Treatment with α-methyldopa decreased the myometrial amount of labeled noradrenaline in both virgin and pregnant animals, irrespective of the gestational age.

Results of isolated organ experiments

Noradrenaline had no effect on the non-pregnant uterus, and the α-methyldopa-treated non-pregnant uterus exhibited a higher spontaneous activity without being more sensitive to α-adrenergic stimulation (Figure 3). On day 7 of pregnancy, noradrenaline evoked weak concentration-dependent contractions in the control group, but more profound contractions in the uteri of α-methyldopa-treated rats. At term pregnancy (day 21), there was no difference in

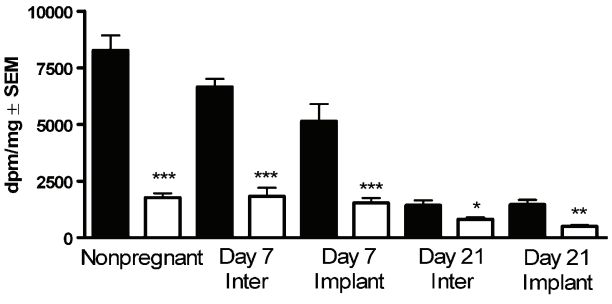
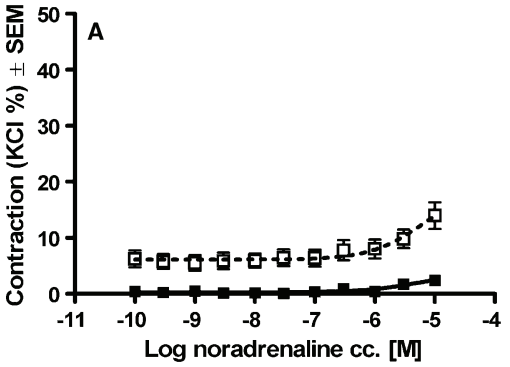
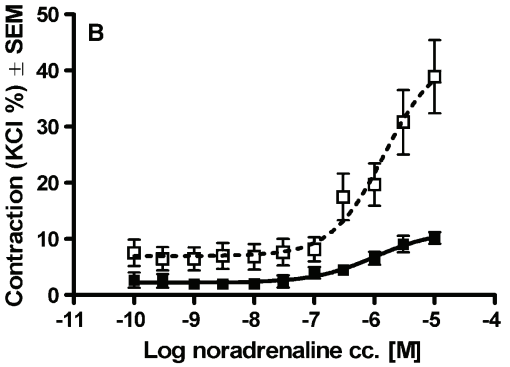


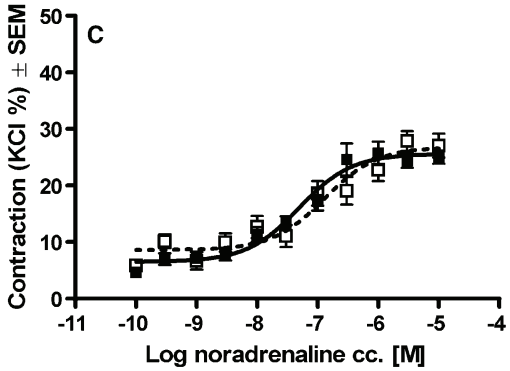
Figure 2. The effects of α-methyldopa treatment on the [^{3}H]noradrenaline-uptake capacity of myometrial samples from virgin and 7- and 21-day pregnant rats. Interimplantation (Inter) and implantation (Implant) sites are presented separately. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the non-treated value. Filled and open columns indicates control values α-methyldopa-treated values, respectively.



	Control	α-Methyldopa-treated	p value
Max. eff. (%)	5.24 ± 2.81	18.50 ± 2.61	n.s.
EC_{50} (μM)	5.99 ± 3.21	6.04 ± 0.63	n.s.



	Control	α-Methyldopa-treated	p value
Max. eff. (%)	12.91 ± 1.48	50.25 ± 6.81	p<0.001
EC_{50} (μM)	1.76 ± 0.679	5.04 ± 1.48	n.s.



	Control	α-Methyldopa-treated	p value
Max. eff. (%)	27.00 ± 1.58	27.64 ± 1.71	n.s.
EC_{50} (μM)	0.341 ± 0.255	0.590 ± 0.245	n.s.

Figure 3. The effects of α-methyldopa treatment on the myometrial contractility evoked by noradrenaline at estrus (A), and on day 7 (B), and day 21 (C). ■ indicates control values, and □ indicates α-methyldopa-treated values. n.s., not significant.

the noradrenaline-evoked contractions between the control and the α-methyldopa-treated groups.

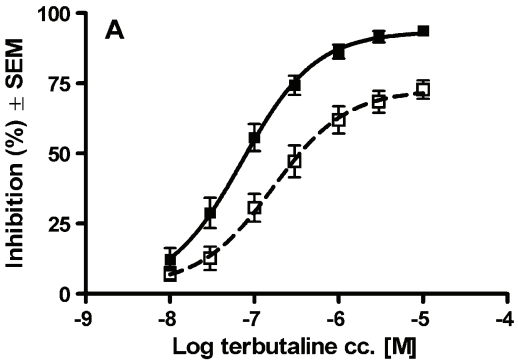
In the next set of experiments, terbutaline inhibited the KCl elicited contractions in a concentration-dependent manner (Figure 4). α-Methyldopa treatment shifted the concentration-response curve of terbutaline slightly to the right, and decreased its maximal effect significantly in the non-pregnant animals. On day 7 of pregnancy, α-methyldopa treatment resulted in a higher maximal effect. At term, α-methyldopa treatment significantly and substantially decreased the relaxant effect of terbutaline, as evidenced by a decreased maximal effect and a higher EC_{50} value.

Results of radioligand-binding experiments

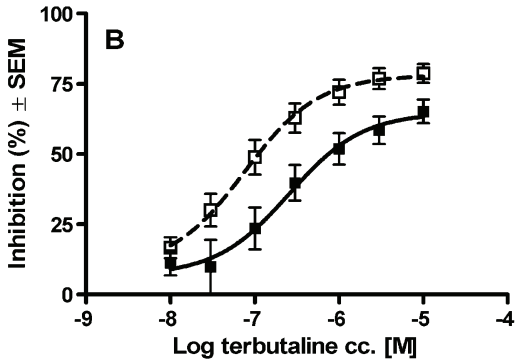
The measured density and affinity values of the saturation plots are presented in Figures 5 and 6. No gestation-dependent change in the density of β-adrenergic receptors was detected, while K_d was increased by the end of pregnancy, and there was a treatment-dependent significant increase on day 21. Concerning the α_2-adrenergic receptors, both B_{max} and K_d were significantly higher on day 7 of pregnancy than in the non-pregnant state, but both parameters had returned to non-pregnant value at term in pregnancy. The only treatment-dependent significant change was a decrease in K_d measured on day 7 of pregnancy.

Discussion

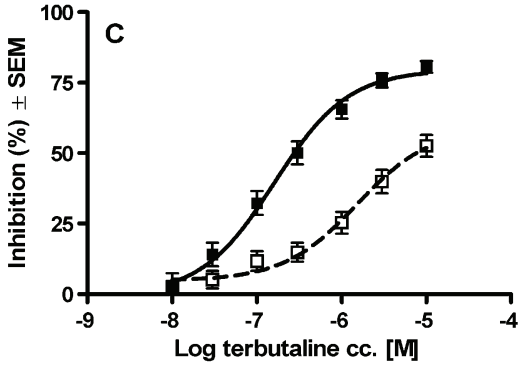
α-Methyldopa, a commonly used agent in the treatment of hypertension during pregnancy, is considered one of the safest drugs because of the vast experience relating to its clinical use (1–3). Despite the large body of clinical experience, and its long use as an antihypertensive, its mechanism of action has been reconsidered several times since the recognition of its antihypertensive effect (17). Earlier works suggested a peripheral site of action, involving either the inhibition of transmitter synthesis or the metabolism to α-methylnoradrenaline, which acts as a 'false' transmitter, thereby decreasing the sympathetic activity (7,8,18). It was later proved that α-methyldopa acts centrally, though peripheral effects were not excluded (19). Next, it was found that the main metabolite of α-methyldopa is a high-affinity agonist of α_2-adrenergic receptors (20). It was also suggested that the metabolites of α-methyldopa may act at almost all types of adrenergic receptors (9). According to the most widely accepted concept, α-methyldopa exerts its antihypertensive action through the stimulation of α_2-adrenergic receptors



	Control	α-Methyldopa-treated	p value
Max. eff. (%)	93.93 ± 1.94	75.74 ± 3.14	p<0.001
EC_{50} (μM)	0.099 ± 0.015	0.482 ± 0.200	p<0.05



	Control	α-Methyldopa-treated	p value
Max. eff. (%)	68.24 ± 4.09	77.27 ± 3.34	p<0.05
EC_{50} (μM)	0.396 ± 0.162	0.128 ± 0.022	n.s.



	Control	α-Methyldopa-treated	p value
Max. eff. (%)	81.14 ± 2.17	64.77 ± 3.27	p<0.001
EC_{50} (μM)	0.252 ± 0.038	1.37 ± 0.265	p<0.001

Figure 4. The effects of α-methyldopa treatment on the relaxation effect of terbutaline at estrus (A), and on day 7 (B) and day 21 (C). ■ indicates control values, and □ indicates α-methyldopa-treated values. n.s., not significant.

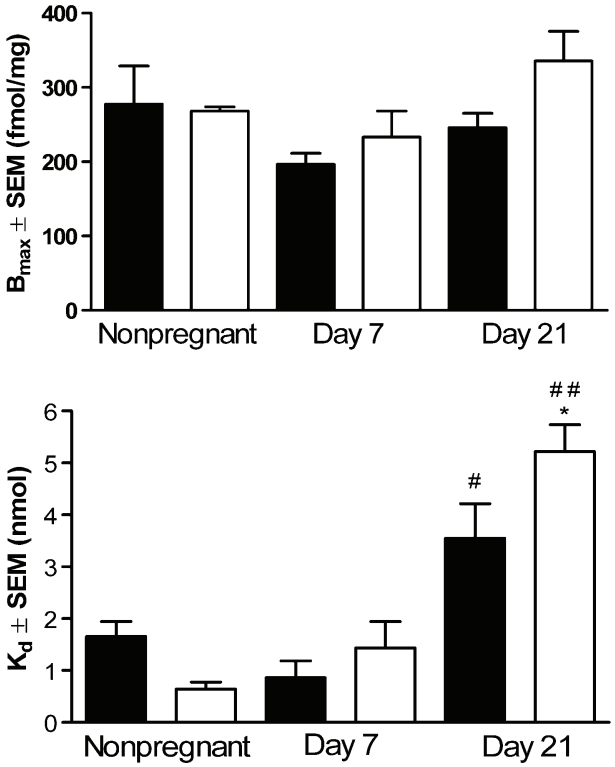


Figure 5. The measured B_{max} and K_d values of β_2-receptors. Filled and open columns indicate control values α-methyldopa-treated values, respectively. $*p < 0.05$ compared with the non-treated value. $\#p < 0.05$ and $\#\#p < 0.01$ compared with the corresponding non-pregnant values.

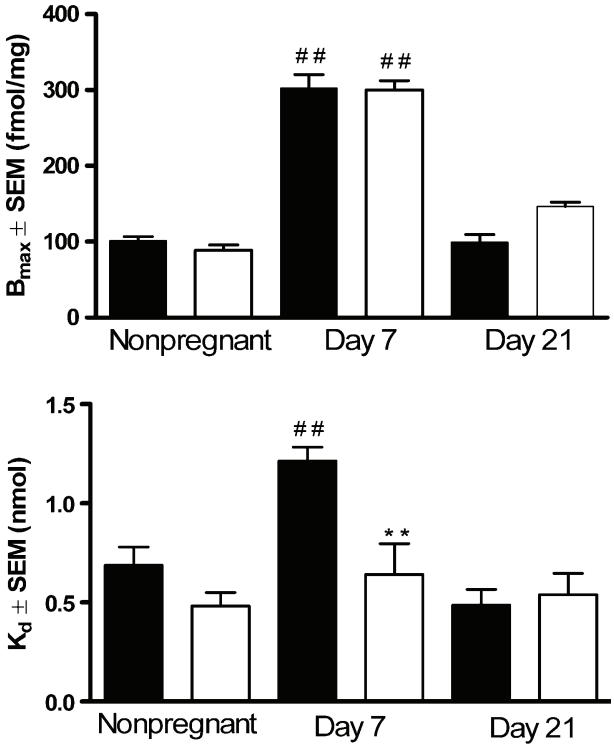


Figure 6. The measured B_{max} and K_d values of α_2-receptors. Filled and open columns indicate control values α-methyldopa-treated values, respectively. $**p < 0.01$ compared with the non-treated value. $\#\#p < 0.01$ compared with the corresponding non-pregnant value.

within the central nervous system, causing a decrease in the efferent sympathetic tone. In spite of several decades of obstetrical use of α-methyldopa, the available information on a direct uterotropic effect is very limited.

Our transmitter liberation results revealed that α-methyldopa all but abolished the electrically evoked noradrenaline peak in the estrus and in the early pregnant state. Implantational and interimplantational sites of the pregnant uterus were processed separately because a large body of evidence suggests that many uterine functions can exhibit site-dependent differences. In the implantational sites of the guinea pig, the pregnancy-induced adrenergic denervation starts earlier, as evidenced by immunohistochemical studies, in good agreement with the activity of tyrosine hydroxylase in the uterine tissue (21,22). These findings were reinforced by our previous observation that the implantation site corresponded to an earlier and more marked decrease in transmitter-uptake capacity and release (14).

In the present experiments, treatment with α-methyldopa was associated with a more marked inhibition of liberation at the interimplantational sites, while in the case of implantational sites only the first stimulation resulted in a significantly smaller amount of liberated noradrenaline.

However, the release is similarly abolished from both α-methyldopa-treated sites on the 7th day of pregnancy, indicating that the difference in the significance of the release pattern is caused by the more marked physiological denervation at the implantational sites. Hence, it could be speculated that α-methyldopa treatment can 'speed up' the physiological degeneration of the adrenergic fibers of the pregnant uterus. The results of the tissue radioactivity also favour this concept. By the time of late pregnancy, this denervation has progressed, leading to the ineffectiveness of electrical stimulation, and thus the α-methyldopa treatment cannot inhibit the release. The mechanism of the transmitter release-inhibiting effect of α-methyldopa is an unanswered question, but it is in good agreement with the finding of Ihalainen and Tanila that the α_2-agonist dexmedetomidin, infused locally, decreased the liberation of noradrenaline in the nucleus accumbens of the mouse (23). Their study proved that this effect is mediated through the α_{2A} type of adrenergic receptors.

The superfusion technique is suitable for characterisation of the presynaptic functions of the transmission, i.e. the transmitter uptake and release

capacity. As far as the postsynaptic surface is concerned, a separate set of experiments was carried out on isolated uterine rings to characterise the changes elicited in the contractility responses by α- and β-adrenergic agonists (noradrenaline and terbutaline, respectively).

The contractions mediated by α-adrenergic receptors were not affected by α-methyldopa in non-pregnant and late-pregnant rats, but were significantly increased in early pregnancy. Treatment resulted in the lower efficacy and potency of terbutaline in non-pregnant and late-pregnant animals, while only limited change was found in the early-pregnant rats. Combining these findings, we concluded the same change for all 3 stages of gestation: the overall effects of sympathomimetics were shifted toward an increased contractility either by a decreased β-receptor-mediated relaxation or by a potentiated α-receptor-mediated contraction. The reasons for these gestational age and treatment-dependent changes in the effects of the adrenergic agonists are not understood.

As high K^+ concentrations depolarise not only the smooth muscles, but also the adrenergic neurons, the liberated noradrenaline may contribute to the contractile effect of KCl. Considering the results of the superfusion experiments, it seems reasonable that this contribution is less in the case of α-methyldopa-treated uterine rings, implying that the tensions elicited by KCl for normalisation of the agonist-induced contractions could exhibit treatment-dependent differences. These differences could be more relevant when the KCl tension is used as an original state which is relaxed by terbutaline. As terbutaline displayed significantly less pronounced effects on the non-pregnant and late-pregnant uteri, it is concluded that this noradrenaline-mediated component of the KCl tension is not crucial in these experiments.

Characteristic pregnancy-dependent changes in the results of radioligand-binding experiments were found in the case of the α_2-adrenergic receptor. Its K_d value showed a maximum in early pregnancy, followed by a decline to its non-pregnant value. α-Methyldopa treatment decreased this maximum, which is in line with our hypothesis, i.e. the treatment can mimic a later phase of the physiological gestation-induced development of the myometrium. The higher affinity of α_2-receptors from early pregnant and treated rats is in good agreement with the increased efficacy of noradrenaline in evoking contractions. However, the role of α_1-receptors in the α-methyldopa-induced higher contractility cannot be excluded.

The mechanism of the decreased relaxing effect of terbutaline in virgin rats and in late pregnancy (day 21) is also a puzzle, though the treatment-dependent decreased affinity of β_2-receptors could be responsible at term.

A large body of experimental data indicates a functional interplay between α_2- and β-adrenergic receptors (24–29). The densities of these receptors change in parallel in the human brain in advancing age and in different pathological states (30). Independently of the underlying mechanism, α-methyldopa-induced alterations in the pharmacological effects of sympathomimetics can be of therapeutic importance, presuming that a similar final effect can develop in the human body. As many over-the-counter preparations contain α-receptor agonists (e.g. nasal decongestants), their usage can seriously increase the uterine contractility when the concomitant pregnancy-induced hypertension is treated with α-methyldopa. On the other hand, the decreased relaxant effect of terbutaline indicates that α-methyldopa can make β-mimetic-based tocolytic therapy less effective, thereby increasing the danger of premature delivery.

As there are no direct data that exclude modulation of the effects of adrenergic agonists similar to our finding, sympathomimetics should be used with great care in pregnant women treated with α-methyldopa.

Acknowledgements

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Szuperfúziós technika alkalmazása a myometriális adrenerg transzmisszió vizsgálatára

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Szegedi Tudományegyetem, Gyógyszerhatástani és Biofarmáciai Intézet[1] (vezető: Prof. Falkay György)
Szegedi Tudományegyetem, Szülészeti és Nőgyógyászati Klinika[2] (vezető: Prof. Pál Attila) közleménye

Összefoglalás: Az adrenerg rendszer egyike a terhes myometrium kontraktilitását meghatározó tényezőknek. Ugyanakkor a rendszer terhesség, farmakológiai beavatkozás vagy a hormonális milió változásának hatásra bekövetkező funkcionális válaszairól kevés adat áll rendelkezésre. Célul tűztük ki az idegtudományban használatos szuperfúziós technika alkalmazását a myometriális adrenerg transzmisszió preszinaptikus oldalának funkcionális vizsgálatára. Nem vemhes és kora vemhes kontroll és α-metil-dopával kezelt patkányokból, ill. eltérő hormonális státuszú betegekből (rendszeres ciklussal rendelkező, ill. postmenopausás) származó myometrium mintákat telítettünk [^{3}H]noradrenalinnal, majd meghatároztuk a szövetek transzmitter felvételét és elektromos stimulációra történő leadását. Azt találtuk, hogy a terhesség indukálta myometriális adrenerg denerváció korábban kimutatható e két paraméter csökkenésével, mint az általánosan alkalmazott hisztokémiai eljárásokkal. Az α-metil-dopa-kezelés szignifikánsan fokozta az adrenerg rostok funkcionális károsodását. Postmenopausás betegek szövetmintái jelentősen kevesebb transzmittert vettek fel és adtak le, mint a rendszeres ciklusú betegekből származók. Megállapítottuk, hogy a szuperfúziós technika alkalmas a myometrium adrenerg rostjainak funkcionális vizsgálatára, az így nyert adatok gyarapíthatják az uterus adrenerg szabályozásáról alkotott tudásunkat.

Kulcsszavak: myometrium, adrenerg rendszer, denerváció, α-metil-dopa, szuperfúzió

Bevezetés

Több mint 30 év telt el azóta, hogy *Raiteri és mtsai* megalkották az első szuperfúziós készüléket, mely lényegét tekintve azóta sem változott. A módszer segítségével meghatározható különböző neurotranszmitterek preszinaptikus oldalról történő felszabadulása. A szerzők eredeti kísérleteikben az eljárást különböző farmakonok transzmitter reuptake gátló, illetve transzmitter-felszabadulást fokozó hatásának megkülönböztetésére használták [1].

A szuperfúziós technika alapelve, hogy a spontán vagy stimulusra felszabaduló transzmittert egy konstans áramlás segítségével azonnal eltávolítjuk a felszabadulás helyéről, ezáltal az nem tud visszakerülni a neuronba reuptake mechanizmussal. Mindebből következik, hogy a módszert elsősorban az idegtudomány alkalmazza különböző transzmissziók preszinaptikus oldalának funkcionális vizsgálatára. A szuperfúziós technika segítségével igen jelentősen bővült a neurotranszmisszióval kapcsolatos tudásunk, többek között így állapították meg, hogy egyes neurotranszmitterek az exocitózis mellett felszabadulhatnak bizonyos körülmények között karriermediált transzporttal is [2]. Idegrendszeri támadáspontú farmakonok hatásmechanizmusának tisztázására is nagyon sokoldalúan használható eszköz a szuperfúzió [3].

A terhes myometrium kontraktilitását alapvetően meghatározza három élettani mediátor-rendszer: a prosztaglandinok, az oxitocin és az adrenerg rendszer [4, 5]. E mecha-

nizmusok jelentőségét illusztrálja, hogy mindhármat tokolitikus célú beavatkozás támadáspontjának tekintjük, jóllehet rutinszerűen csak β-adrenerg receptoron ható szimpatomimetikumok terjedtek el a klinikai gyakorlatban [6]. Az adrenerg rendszer sajátossága, hogy a transzmitter – a noradrenalin – mind innerváció, mind pedig a véráram útján elérheti receptorát. A uterus terhesség alatti drámai változásai jól ismertek, köztük a myometrium beidegzésének fokozatos élettani degenerációja, ami feltehetően hozzájárul a simaizomzat tartósan relaxált állapotához. Elsőként a terhes uterus adrenerg rostjainak denervációját ismerték fel [7], mára azonban tisztázott, hogy hasonló változáson esik át a kolinerg és a peptiderg beidegzés is [8]. Ennek ellenére máig az adrenerg rostok degenerációját tartjuk a terhesség indukálta myometriális denerváció legfontosabb elemének. A jelenségre vonatkozó szakirodalom szinte kizárólag biokémiai, ill. morfológiai tanulmányokból áll, funkcionális vizsgálatok alig történtek [9, 10]. Jelen vizsgálatsorozatunk célja annak eldöntése volt, hogy szuperfúzió alkalmas-e a myometrium adrenerg innervációjának funkcionális vizsgálatára, azzal kimutatható-e a terhességi denerváció, valamint hogy a módszer érzékenység tekintetében hogy viszonyul az eddig alkalmazott hisztokémiai eljárásokhoz.

A hypertonia az egyik leggyakoribb terhességi komplikáció, az esetek 7–9%-ában jelentkezik, és igen jelentős mértékben járul hozzá a maternális és perinatális morbiditási és mortalitási mutatókhoz [11, 12]. Kezelésére – a hatékonysági és biztonsági profilja, valamint a felhalmozódott évtizedes tapasztalat miatt – a mai napig elsőként választandó a centrálisan ható α-metil-dopa [13]. A szer az adrenerg neuronban belép a katekolaminok bioszintézisébe, a belőle képződő α-metil-noradrenalin α_2-adrenerg agonistaként hatva csökkenti a szimpatikus tónust, így a vérnyomást is [14]. Annak eldöntésére, hogy centrális szimpatikus tónus csökkentésével ható szer milyen hatást gyakorol a myometriális adrenerg funkciókra, kísérleteinket kiterjesztettük α-metil-dopával kezelt nem terhes és kora terhes állatok uterusmintáira.

A továbbiakban megbizonyosodtunk a módszer alkalmazhatóságáról a humán myometrium esetében is, majd az előkísérletek után megvizsgáltuk különböző hormonális státuszú – aktív ciklussal rendelkező ill. menopausán átesett – betegekből származó myometriumminták noradrenalin-felvételét és stimulációra történő leadását.

Anyagok és módszerek

Kísérleti állatok

A vizsgálathoz használt 200–250 g-os, nőstény Sprague-Dawley patkányok pároztatása hajnalban történt, speciális ketrecben. A megtermékenyülést a natív vaginális kenetben történő spermium detektálásával bizonyítottuk, a megtermékenyülés napját tekintettük a vemhesség első napjának. Az állatokon CO_2 kamrában végeztünk eutanáziát. A nem vemhes állatok mind ösztrusz állapotban voltak, ezt szintén natív vaginális kenetből állapítottuk meg. Az uterus szövetéből mintákat metszettünk, a vemhes állatok esetében pedig különbséget tettünk az implantáció helyéről származó, és az interimplantációs helyekről származó minták közt. A vemhesség során a 7., 14. és 21. napon végeztünk kísérletet, ezek a 22 napos gesztáció egyes harmadjainak végét reprezentálják. A patkányok egy részét a kísérlet előtt α-metil-dopával kezeltük intraperitoneálisan, napi 200 mg/kg adagban, egy héten át.

Humán szövetminták

A humán minták olyan páciensekből származtak, akik különböző okokból – pl.: myoma, cervix carcinoma – histerectomián estek át, és beleegyeztek az eltávolított szövet tudományos célú felhasználásába. A szövetekből csak a makroszkóposan intakt részeket használtuk. A humán minták esetében, a szövet eltávolítása és a kísérlet kezdete között legfeljebb 25 perc telt el, a szövet szállítása de Jongh pufferben 5°C-on történt.

[^{3}H]noradrenalin-felvétel és -felszabadulás meghatározása

A továbbiakban a humán és patkányból származó minták esetében azonos módon jártunk el. A mintákat (20–30 mg) megtisztítottuk a kötőszövettől és az endometriumtól, felaprítottuk, és oxigenizálás közben 10^{-7} M [^{3}H]noradrenalinnal inkubáltuk (Perkin Elmer Life Sciences, Boston, USA; specifikus aktivitás: 7,94 Ci/mmol) 37°C-on 60 percig. Ezután háromszor mostuk de Jongh pufferrel, majd a szövetdarabokat szuperfúziós tartályba helyeztük (Experimetria Kft, Budapest,), ahol 1 ml/min-es folyamatos áramlást biztosítottunk 60 percig de Jongh pufferral. Annak érdekében, hogy a jelzett noradrenalin ne metabolizálódjon és a teljes liberálódott mennyiség a szuperfuzátumba kerüljön, a puffer tartalmazott monoamin-oxidáz gátló pargilint, noradrenalin-reuptake gátló dezipramint és extraneuronális uptake gátló dezoxikortikoszteront (10^{-5} M koncentrációban mindegyikből). A puffer összetétele: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4 és 6 mM glükóz, pH 7,4. A puffert a kísérlet alatt végig 37°C-on tartottuk, és folyamatosan oxigenizáltuk 5% (v/v) CO_2-ot tartalmazó oxigénnel. Egy 60 perces kimosási periódus után 3 perces frakciókat gyűjtöttünk, összesen 22-t. A kísérlet végén a szöveteket szolubilizáltuk 1 ml Solvable (Canberra-Packard Kft, Budapest) segítségével 3 órán át 60°C-on. Az egyes 3 perces frakciók, és szöveti szolubilizátumok [^{3}H] tartalmát folyadék szcintillációs spektrofotométerrel határoztuk meg. A transzmitter felszabadítására elektromos térerő ingerlést alkalmaztunk, melyeket egy programozható stimulátor (Experimetria Kft, Budapest) segítségével idéztük elő az 5. és a 15. frakció alatt. Az egyes stimulációs periódusok 360 jelből álltak, és paramétereik megfeleltek a szelektív idegingerlés jellemzőinek (feszültség, 40 V; jelszélesség, 2 ms; frekvencia, 2 Hz). A frakciók [^{3}H]noradrenalin tartalmát frakcionált release-ben fejeztük ki, ami a felszabadult, izotóppal jelölt transzmitter mennyiségét jelenti egy 3 perces frakcióban a teljes aktuálisan jelen lévő rádióaktivitás százalékában kifejezve. Minden további vegyszert a Sigma-Aldrich Kft-től rendeltük (Budapest). Minden kísérleti protokoll megfelelt az etikai, illetve állatkísérleti etikai normáknak, az elvégzésükhöz szükséges engedélyekkel rendelkezünk.

Statisztikai értékelés

Az eredmények statisztikai elemzésére kétmintás t-próbát, ill. varianciaanalízist használtunk, a teszteket GraphPad Prism 2.01 (Graph Pad Software, San Diego, USA) szoftverrel végeztük. Minden bemutatott adat legalább 6 független kísérlet eredményeinek átlagából származik.

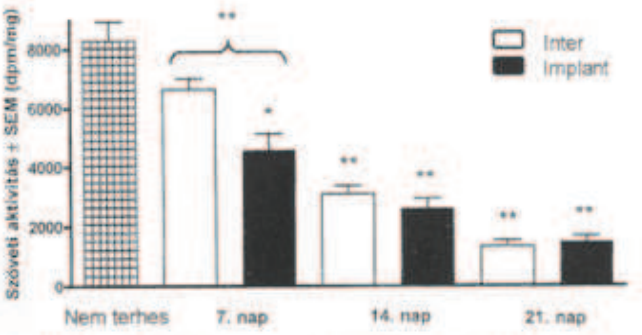
Eredmények

A patkánykísérletek eredményei

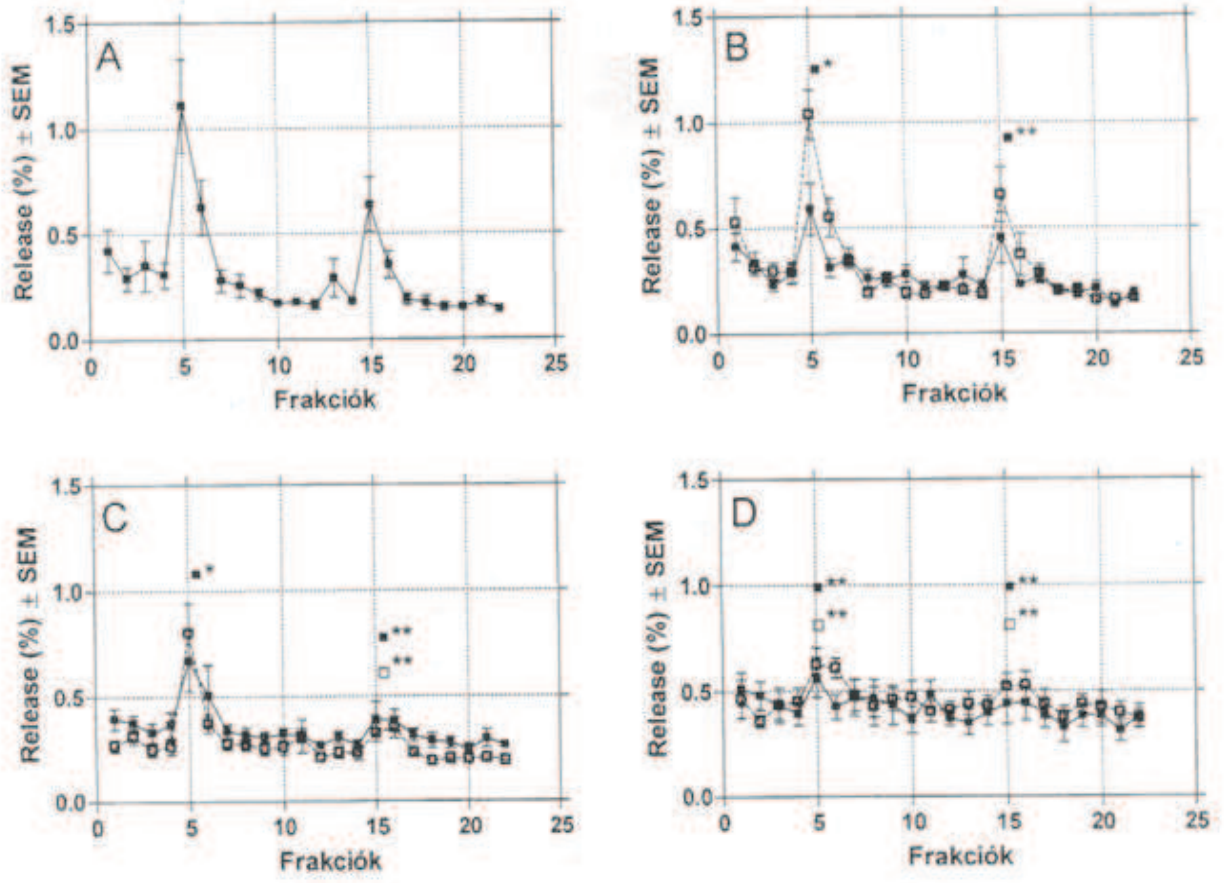
A minta transzmitterfelvevő kapacitásának jellemzésére a szöveti aktivitást használtuk, ezt dpm/mg szövetben fejeztük ki. Azt találtuk, hogy ez az érték a nem terhes állapotban volt a legmagasabb, és fokozatos csökkenés volt tapasztalható a terhesség előrehaladtával *(1. ábra)*. A hetedik napon, az implantáció helyéről származó mintákban a transzmitterfelvétel szignifikánsan alacsonyabb volt, mint az implantációk közötti helyekről származó minták esetében. A további napokon nem találtunk implantációfüggő eltérést, ám a szöveti aktivitás fokozatosan tovább csökkent.

A nem vemhes állatokban jelentős mennyiségű [^{3}H]noradrenalin szabadult fel elektromos stimuláció hatására *(2. ábra)*. A kísérlet során kétszer ingereltük a szövetet, hogy képet kapjunk a vizsgált szövet noradrenalin-felszabadító kapacitásáról. A második inger minden esetben kisebb transzmitter felszabadulást eredményezett, mint az első. A myometriumban a terhesség előrehaladtával – a szöveti aktivitáshoz hasonlóan – az elektromos ingerléssel kiváltható noradrenalin felszabadulás fokozatosan csökkent. A vemhesség első harmadának végére (7. nap, B panel) az implantációk helyéről származó minták transzmitter felszabadulása szignifikánsan alacsonyabb volt, mint a nem vemhes myometriumból mért felszabadulás. A 14. vemhességi napra már az implantációk közötti terület is alacsonyabb transzmitter csúcsot adott, ez a tendencia a terminusra tovább fokozódott. Általánosságban azt láthatjuk, hogy a második elektromos erőtéringerléssel kiváltott liberáció érzékenyebben reagált a noradrenalin-felszabadulás változásaira, mint az első.

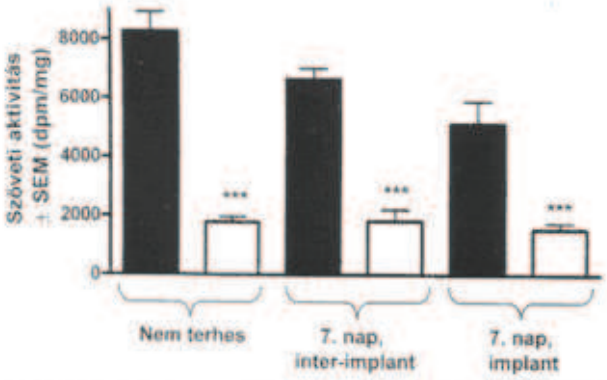
Kísérleteink szerint az egyhetes α-metil-dopával történt kezelés igen markánsan csökkentette mind a [^{3}H]noradrenalin adrenerg rostokba történő felvételét, mind pedig stimulációra történő leadását *(3-4. ábra)*.



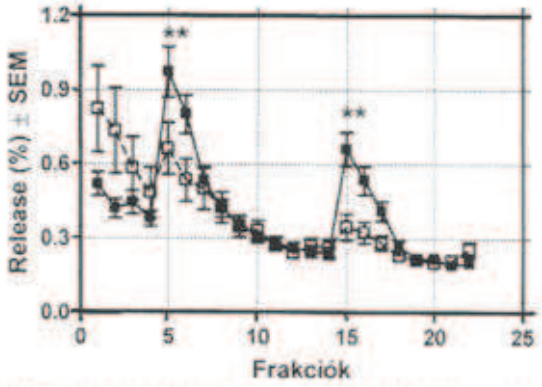
*1. ábra. A patkány myometrium [^{3}H] noradrenalin-felvevő kapacitása a terhességi idő függvényében. A terhesség alatt külön értékeltük az implantáció helyéről származó mintákat (■) és az implantációk közötti helyekről származókat (□). * és **: p<0,05 és a p<0,01 a nem terhes állapothoz viszonyítva*



*2. ábra. Elektromos térerő ingerlés által kiváltott [^{3}H] noradrenalin-felszabadulás a patkány myometriummintákból ösztruszban (A), a terhesség 7. (B), 14. (C) és a 21. napján (D). B–D paneleken: ■ az implantáció helyéről származó mintákat, míg a □ az implantációk közötti helyekről származó mintákat jelöli. * és **: p<0,05 és p<0,01 a nem terhes állapothoz viszonyítva*



*3. ábra. A myometrium [³H] noradrenalin felvevő kapacitása kontroll (■) és α-metil-dopával kezelt (□) nem terhes és 7 napos terhes patkányokból, utóbbi esetben külön az inter-implantációs és implantációs területekről. ***: p<0,001 a két csoport között*

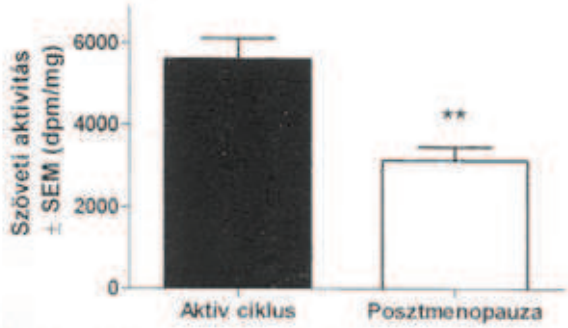


*5. ábra. Elektromos térerő ingerlés által kiváltott [³H] noradrenalin felszabadulás humán myometrium mintákból. ■ : rendszeres menstruációs ciklussal rendelkező betegekből származó minták, □: postmenopausában lévő betegek mintái; **: p<0,01 a két csoport között*

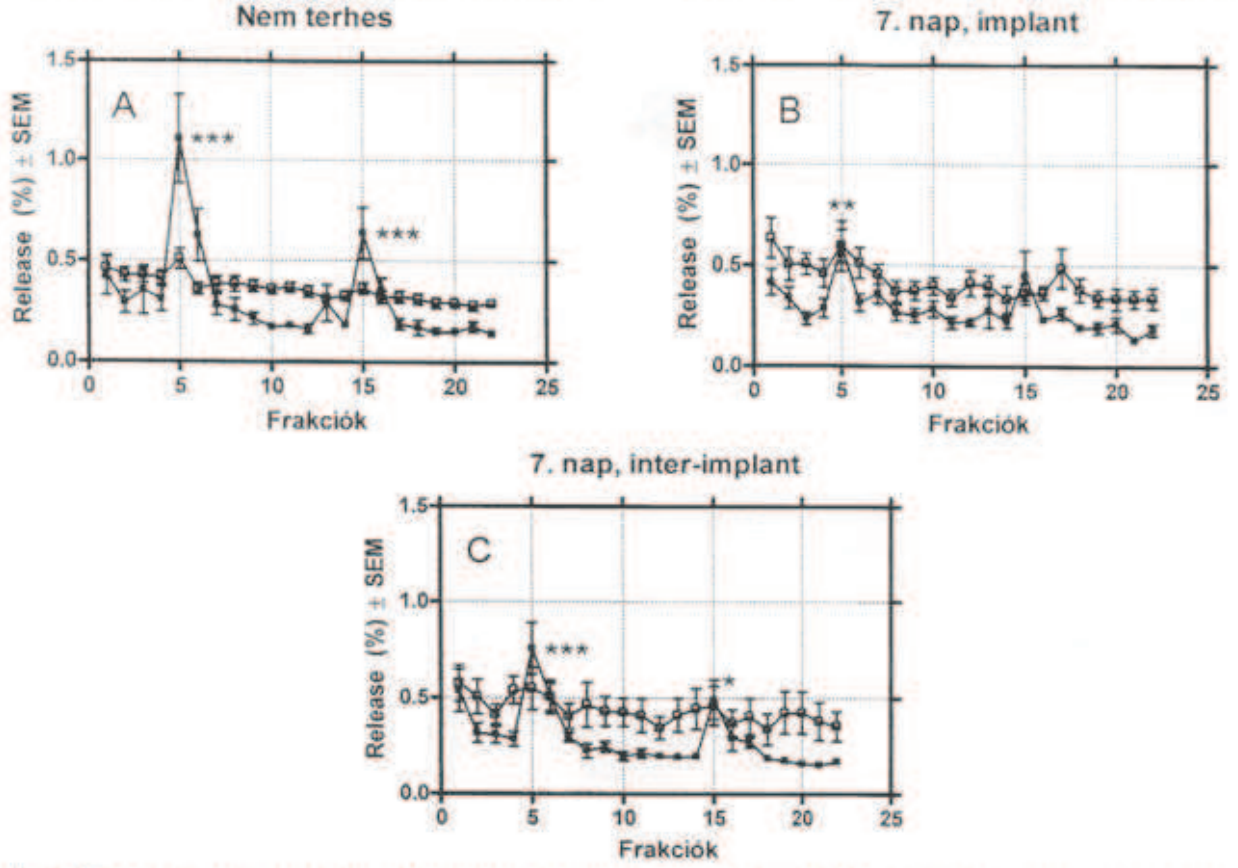
A kezelés mindkét paramétert már terhesség előtt, ill. az első harmadban a terminusra jellemző értékre módosította.

A humán myometriumon végzett kísérletek eredményei

Kísérleti elrendezésünkben sikerült megmérnünk a humán myometriumból történő [³H]noradrenalin-felszabadulást. A rendszeres menstruációs ciklussal rendelkező páciensek esetében elektromos stimulusra jól mérhető transzmitter felszabadulást regisztráltunk *(5. ábra)*. Hasonlóan az állatkísérletes eredményekhez, a második



*6. ábra. A humán myometrium [³H]noradrenalin-felvevő kapacitása aktív ciklusú betegek (■) és postmenopausában lévő nők (□) myometriumában. **: p<0,01 a két csoport között*



*4. ábra. Elektromos térerő ingerlés által kiváltott [³H] noradrenalin-felszabadulás kontroll (■) és α-metil-dopával kezelt (□) patkányok myometriummintáiból nem terhes állapotban (A) és a terhesség 7. napján, az implantátumok helyén (B), ill. az implantátumok közötti területről (C). *, ** és ***: p<0,05, p<0,01 és p<0,001 a két csoport között*

stimulus által kiváltott transzmitter csúcs kisebb, mint az első. A prezentált eredmények 11 rendszeres ciklusú beteg myometriumából származnak, az alacsony elemszám miatt a csoporton belül nem vizsgáltuk a hormonális státusz esetleges hatását a szuperfúziós technikával meghatározott paraméterekre. A posztmenopauzában lévő páciensek – 5 beteget involváltunk – mintáiból a [^{3}H]noradrenalin-felszabadulás szignifikánsan kisebb volt a rendszeres ciklusú páciensek eredményeihez viszonyítva. Az állatkísérletek eredményeihez hasonlóan a transzmitterfelszabadulás és a szöveti aktivitás párhuzamosan változtak: a postmenopausában lévő páciensek myometriumának noradrenalin-felvevő kapacitása szignifikánsan kisebb volt *(6. ábra)*.

Megbeszélés

Vizsgálataink alapján megállapítható, hogy a szuperfúziós technika alkalmazható a myometriális adrenerg transzmisszió preszinaptikus funkcióinak vizsgálatára. A módszer alkalmasságát mind patkány, mind pedig humán myometrium-mintákon demonstráltuk. A mért paraméterek – transzmitterfelvétel és stimulációra történő leadás – szignifikáns különbségeket mutattak a gesztációs idő, farmakológiai beavatkozás, ill. a hormonális státusz függvényében.

A terhesség indukálta adrenerg denerváció funkcionális vizsgálatára alkalmas a szuperfúzió, bizonyos tekintetben árnyaltabb képet kaphatunk a folyamatról, mint az általánosan használt morfológiai-hisztokémiai eljárásokkal. Míg az utóbbi módon vizsgálva a terhesség utolsó harmadában detektálható az adrenerg rostok jelentős károsodása, addig a szuperfúzióval meghatározott paraméterek már az első harmad végén – a 7. napon – szignifikáns mértékben mutatják az adrenerg neuronok csökkent funkcióját [15]. A gesztáció első és második harmadának végén jelentősebb funkcionális károsodást detektáltunk az implantációs területekről, mint az azok közötti régiókból. Ezzel funkcionálisan is megerősítettük azt a korábban leírt tényt, mely szerint az adrenerg denerváció az implantációk körül kezdődik, és terminusig ott a legkifejezettebb [16]. A szuperfúziós technika hátrányaként ugyanakkor megemlítendő, hogy a szövettani módszerekkel ellentétben a feldolgozott mintán belül nem képes struktúrák között különbséget tenni. Így az uterus denervációján belül nem különíthető el a myometriális vaszkulatúrát, ill. magát a myometriumot érintő adrenerg neuron degeneráció [8, 17].

Az évtizedes antihipertenzív célú használat ellenére az α-metil-dopa hatásmechanizmusa nem ismert teljes mértékben. A legkorábbi tanulmányok a szer hatását a katekolaminok bioszintézisének gátlására vezetik vissza, ami egyben a perifériás támadáspontra is utal [18]. Később került az előtérbe az α-metil-noradrenalin által mediált centrális hatás, a „hamis transzmitter" elmélet [19]. A ma legelfogadottabb elképzelés szerint perifériás hatáskomponens nem zárható ki a szer elsődleges centrális támadáspontja mellett, amiért főleg az α-metil-noradrenalin α_2-adrenerg receptoron kifejtett agonista hatása felelős [14, 20]. A kiterjedt szülészeti felhasználás ellenére nem vizsgálták az α-metil-dopa direkt uterotróp hatását. Jelen eredmények azt mutatják, hogy a szer gátolja a myometriális adrenerg transzmisszió preszinaptikus funkcióit, ezáltal potenciálja a terhesség általi élettani adrenerg denervációt. A szuperfúziós technika nem ad információt az észlelt jelenség posztszinaptikus oldalra gyakorolt hatásáról, ám kézenfekvő, hogy az élettaninál jelentősebb, ill. korábbi transzmitterhiány fokozza a receptorok denzitását és érzékenységét. Amennyiben így van, és a humán szövetben is hasonló összefüggések érvényesülnek, felvetődik az exogén szimpatomimetikumokra – pl. tokolitikumokra vagy dekongesztánsként alkalmazott receptoragonistákra – adott markánsabb myometriális válasz. Utóbbi esetben pedig az emelkedett kontraktilitás a koraszülés fokozott kockázatát is jelentheti, ám erre vonatkozó klinikai adatok még nem állnak rendelkezésre.

A módszer alkalmazhatóságát humán szövetmintán is igazoltuk, két markánsan eltérő hormonális státuszú betegcsoport – aktív ciklussal rendelkező és menopausán átesett nők – szuperfúzióval meghatározható paraméterei között szignifikáns eltéréseket találtunk. A postmenopausás myometrium-minták kevesebb jelzett noradrenalint vesznek fel és adnak le, ami részben degenerálódott adrenerg innervációra vagy a rostok csökkent funkciójára utal. Korábbi – hisztokémiai módszerekkel végzett – állatkísérletes vizsgálatok szerint az ösztradiolkezelés éppen ellentétes hatást vált ki, csökkenti a patkány uterus noradrenalin-tartalmát, ami alapján várható lenne az ösztrogénnel alulexponált, postmenopausás myometrium emelkedett noradrenalin-felvétele és -leadása. [21, 22]. Ugyanakkor *Brauer és mtsai* kísérleteikhez infantilis állatokat használtak, melyek plasztikusan reagálnak az elvégzett beavatkozásokra. A speciesbeli különbségeken túl ezért sem célszerű e korábbi állatkísérletes adatok és a prezentált eredmények között párhuzamot vonni.

Mindezek alapján megállapítható, hogy a szuperfúziós technika olyan funkcionális információkat adhat a myometrium adrenerg transzmissziójával kapcsolatban, melyek kiegészítik, árnyalják a jóval elterjedtebb szövettani módszerekkel kapott eredményeket. Az eljárás jelentősen gazdagíthatja a myometriális működést érintő kórállapotokról, ill. a szülészeti praxisban rutinszerűen alkalmazott farmakonok direkt myometriális hatásairól alkotott tudásunkat.

Köszönetnyilvánítás

Jelen vizsgálatokat az OTKA 062707 számú projektjének támogatásával végeztük. Zupkó István Bolyai János Kutatási Ösztöndíjban részesül. A szerzők a támogatásokért köszönetüket fejezik ki.

Irodalom

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Csonka D, Kormányos Zs, Csík G, Zupkó I, Falkay G.: *Utilization of superfusion technique for the investigation of myometrial adrenergic transmission*

Adrenergic system is one of the crucial factors determining the contractility of the pregnant myometrium. However, results concerning the functional changing of the system as a consequence of pregnancy or pharmacological intervention are limited. The aim of the present study was application of superfusion technique for the functional investigation of myometrial adrenergic transmission. Uterine samples from virgo and early pregnant control or α-methyldopa-treated rats as well as tissues from patients of different hormonal states were saturated with [^{3}H]noradrenaline and then the uptake capacity and the electrically-evoked release were determined. Pregnancy-induced adrenergic denervation can be detected earlier by superfusion than histochemical methods. Treatment with α-methyldopa increased the functional deterioration of the adrenergic fibers. Postmenopausal status decreased transmitter uptake and release compared to regular menstruation. It is concluded that superfusion is suitable and reliable tool for functional investigation of myometrial adrenergic transmission.

Keywords: myometrium, adrenergic system, denervation, α-methyldopa, superfusion

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