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

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 pre-synaptic 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 [^3H]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, Nosstrom & 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 [^3H]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 [^3H]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 [^3H] 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 [^3H]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-[^3H](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 [^3H]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 [^3H]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 [^3H]noradrenaline release

EFS evoked a substantial [^3H]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 [^3H]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 [^3H]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 [^3H]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 [^3H]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 [^3H]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 [³H]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 [^3H]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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Received 19 May 2005

First decision 23 June 2005

Revised manuscript received 9 August 2005

Accepted 16 August 2005