THE EFFECTS OF ADJUVANT ARTHRITIS ON THE MYOMETRIAL ADRENERGIC FUNCTIONS IN THE NONPREGNANT AND THE LATE-PREGNANT RAT

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The beneficial effects of pregnancy on the symptoms of inflammatory diseases are well documented. The modulation in the uterine functions in the presence of generalized inflammation, however, is much less characterized. The aim of the present study was to explore the modulatory action of adjuvant arthritis on the adrenergic functions of the uterus in nonpregnant and late pregnant rats. Adjuvant arthritis was induced by the subplantar injection of M. butyricum. Presynaptic functions were characterized by a superfusion technique and by registration of the contractions of isolated uterine rings elicited by electric field stimulation. The functions of the adrenoceptors were characterized by constructing concentration-response curves with agonists for both α- and β-receptors. Where these curves differed significantly from the control, the expressions of these receptors at the mRNA level were additionally determined. Adjuvant arthritis substantially decreased the uptake and release of [3H]noradrenaline in myometrial samples from nonpregnant rats, but caused no change at term. The electrically induced contractions were decreased by inflammation in both gestational states. Arthritis resulted in decreased β-adrenoceptor-mediated relaxation (in both the nonpregnant and the late-pregnant animals) and an increase in α-mediated contraction at term. It can be concluded that adjuvant arthritis deteriorates the adrenergic innervation of the uterus. The effects of exogenous sympathomimetics are shifted, favoring a state of higher contractility. If similar mechanisms are operative in humans, the present results could imply that β-adrenoceptor agonists are not ideal tocolytics when pregnancy is complicated by generalized inflammation.

Key words: adjuvant arthritis, adrenergic system, alpha adrenoceptors, beta adrenoceptors, pregnancy, rat myometrium
the animal kingdom and in humans, ranging from a temporary functional deterioration to life-threatening states (15, 16).

It is widely accepted that proinflammatory cytokines and chemokines (interleukin IL-1β, IL-6, IL-8 and tumor necrosis factor alpha) are involved in preterm labor (17). It has recently been proposed that cytokines also play a fundamental role during the physiological birth process, representing an inflammation-like response, and influence the regulation of uterine contractility (18). Besides oxytocin and the prostaglandins, the adrenergic system is responsible for coordination of the myometrial motor activity during pregnancy and labor. The adrenergic system plays a crucial role in regulation of the contractility of the uterine smooth muscle (19-22). However, the uterine smooth muscle itself has been only poorly investigated in RA.

Accordingly, the aim of our study was to determine the changes in the uterine functions in pregnancies complicated by RA. The presynaptic stage of adrenergic transmission, and noradrenaline uptake and electrically induced liberation were investigated by means of a superfusion technique. We analyzed the postsynaptic stage of adrenergic transmission in the uterine smooth muscle, and also the effects of direct neuronal stimulation with an electric field. The possible postsynaptic consequences were investigated in isolated organ experiments in which the myometrial smooth muscles were stimulated with adrenergic agonists. To approach the underlying mechanism, the state of the targeted receptors was characterized through their expression at the mRNA level by means of RT-PCR.

**MATERIALS AND METHODS**

**Animals and chemicals**

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 to be the first day of pregnancy. The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee (registration number: IV/18/1-2002). The animals were sacrificed by CO2 inhalation. If otherwise not specified, substances were purchased from Sigma-Aldrich, Budapest, Hungary.

**Superfusion technique**

**Preparation of the tissues**

Pregnant and nonpregnant rats were euthanized in a CO2 chamber. 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, and they were minced and incubated with 10^5 M [3H]noradrenaline at 37°C for 60 min. The samples were then washed three times with de Jongh buffer containing the monoamine oxidase inhibitor paraglyne, 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 CaCl2, 1 mM MgCl2, 12 mM NaHCO3, 4 mM Na2HPO4 and 6 mM glucose, pH 7.4. The solution was maintained at 37°C and equilibrated throughout the experiment with O2 containing 5% (v/v) CO2. 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 solubilized in 1 ml Solvable (Canberra-Packard, Budapest, Hungary) for 3 hours at 60°C. The [3H] 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 (40 V; pulse width, 2 ms; 2 Hz; these parameters are suitable for neuronal stimulation).

The [3H]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 in dpm/mg tissue.

**Isolated tissue studies**

1. **Preparation of the tissues**

Uterine rings were taken from the uterine horns of pregnant or nonpregnant, treated or nontreated 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 to and maintained at 37°C, and O2 containing 5% (v/v) CO2 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 Ltd., Budapest, Hungary) and recorded with an Isosys Data Acquisition System (Experimetria Ltd., Budapest, Hungary). The areas under the curves were analyzed for a 5-min period after each administration of the tested substances.

2. **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 β-adrenergic receptors (β-ARs). During the equilibration period (90 min), the buffer in the chambers was changed every 15 min, i.e. a total of 6 times. After equilibration, noradrenaline was added to the chamber cumulatively, in a total of 11 doses. 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 characterize the effects of the inflammation on the β-AR-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^{-6}-10^{-4} 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 and the maximal effect and EC_{50} values were calculated with the aid of GraphPad Prism 4.03 (GraphPad Software, San Diego, USA).
Samples (GeneID: 81822). The PCR was performed with a PCR RNase-free distilled water.

RT-PCR studies

Animals were killed in a CO₂ chamber, and uterine samples were collected within 10 min, quickly cleared from connecting tissue, frozen and maintained at -70°C until RNA isolation. PCR studies were made only on the days of pregnancy where the isolated tissue studies revealed the most pronounced changes between the uterine functions of the treated and control animals. Accordingly, for the α₁- and β₂-AR RT-PCR studies, we used the tissue samples of nonpregnant animals. Total RNA was extracted from all collected tissues with acid guanidinium thiocyanate-phenol-chloroform (24). After precipitation with isopropanol, the RNA was washed three times with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 µL DNase and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbances at 260 nm. The tissue activity at any site.

Electric field

Uterine field were taken and mounted in a tissue bath in the same way as in the case of isolated tissue studies. EFS was applied to the mounted tissue 3 times in order to acquire information on the exhaustion. The parameters were as follows: 40 V, duration of EFS: 3 min, pulse width: 0.6 ms, period time: 50 ms, and resting period between EFSs: 3 min. These parameters fit the requirements for selective neuronal stimuli (23). 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 the EFSs were expressed as a percentage of the KCl-evoked contractions. The electrical excitability of the tissues was expressed as the ratio of the areas under the curve of the stimulation period and the curve of their respective poise period.

All of the presented results relating to tissue organ contractilities are the averages of the data from at least 5 independent experiments.

Statistical analysis

All data regarding superfusion and contractile responses are presented as the means of at least five independent experiments; data for RT-PCR experiments and contractions elicited by EFS were evaluated by one-way ANOVA followed by Newman-Keuls post test. Unpaired t-tests were used to test the significance of the effects of AA on EC₅₀ values, maximum responses on the concentration-effect curves and on PCR products. All statistical analyses were performed using GraphPad Prism 4.

RESULTS

Superfusion study

Tissue activity

The tissue activity (expressed in dpm/mg tissue) was used to describe the uptake capacity of the sample for [³H]noradrenaline. This parameter for the uterus from virgo animals was significantly decreased by experimental inflammation (Fig. 1). The activities of the implantation and interimplantation areas of the pregnant uterus were determined separately. It was found that the tissue activity at term declined substantially on both sides, but in contrast with the nonpregnant state, generalized inflammation did not cause any change in activity at any site.

Table 1. Primer pairs used for PCR of α₁- and β₂-adrenoceptors (α₁-AR and β₂-AR) from rat myometrium, the Genebank access numbers, the length of PCR products and the parameters of the multiplication.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Primer sequence</th>
<th>Gene ID</th>
<th>Product size (bp)</th>
<th>Coupling temp. (°C)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁-AR</td>
<td>5'-GTA GCC AAG AGA GAA AGC CG-3'</td>
<td>29412</td>
<td>212</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>5'-CAA CCC ACC ACC ATG CCC AG-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁b-AR</td>
<td>5'-GCT CCT TCT ACA TCC CGC TCG-3'</td>
<td>24173</td>
<td>301</td>
<td>54</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>5'-AGG GGA GCC AAC ATA AGA TGA-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁ρ-AR</td>
<td>5'-CGT GTG CTC CTT CTA CCT ACC-3'</td>
<td>29413</td>
<td>304</td>
<td>53</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>5'-GCA CAG GAC GAA GAC ACC CAC-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β₂-AR</td>
<td>5'-TCT TCG AAA ACC TAT GGG AAC GCC-3'</td>
<td>24176</td>
<td>343</td>
<td>54</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5'-GGA TGT GCC CCT TCT GCA AAA TCT-3'</td>
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</table>
Electrically stimulated [3H]noradrenaline release

EFS evoked maximal [3H]noradrenaline release from the uteri of the nonpregnant control animals (Fig. 1). Two EFSs were applied (in fractions 5 and 15) to obtain information on the release capacity of the tested tissues. The second stimulus resulted in a smaller transmitter peak than the first. In the nonpregnant myometrial tissues, a 14-day history of AA caused a marked and statistically significant decrease in the transmitter release evoked by EFS. Both of the peaks were practically abolished by AA (Fig. 1). On day 21 of pregnancy, the [3H]noradrenaline release evoked by EFS was substantially lower than that before pregnancy and this minimal liberation was not significantly changed in the presence of inflammation (Fig. 1).
Electric field stimulation

Three sets of electric stimuli were administered in order to evaluate the fatigue of the myometrial ring. Significant differences were not observed between the excitabilities evoked by the 3 stimuli. In the nonpregnant animals, inflammation significantly decreased the contractions evoked by EFS (Fig. 2).

By term, this inflammation-related difference in excitability had decreased and only the result of the third stimulation differed significantly.

Contraction and relaxations induced by agonists

Agonist-induced changes in motor activity were investigated via the cumulative dose-response curves of sympathomimetics acting on the α- or β-ARs. The myometrium of nonpregnant animals proved to be practically nonresponsive to α1-adrenergic stimulation, by noradrenaline in the presence of propranolol (10^-6 M). Uterine rings from M. butyricum-treated rats did not exhibit an altered contractility. On the other hand, the relaxant effect of terbutaline was significantly less pronounced, as evidenced by a higher calculated EC50 value, but no change in the maximal effect (Fig. 3).

In late pregnancy α-AR stimulation resulted in substantially higher motor activity, which was further increased by AA. On the other hand, the relaxant effect of terbutaline was slightly decreased as compared with the results from nonpregnant samples, and this relaxation was blunted, i.e. the maximal effect was decreased while EC50 was increased, in the presence of inflammation (Fig. 3).

RT-PCR studies

As the aim of the PCR studies was to find a possible explanation for the AA-related changes in the agonist-induced contractility profile, these studies were restricted to the conditions under which significant differences were exhibited. Therefore, the expressions of the ARs (all types of α1 and β2) at the mRNA level were determined on nonpregnant and late-pregnant uteri.

In the nonpregnant animals, the α1A-AR expression did not exhibit any AA-dependent difference (Fig. 4). The amount of α1B subtype mRNA was markedly increased in the myometrium of the M. butyricum-treated rats, while the α1D subtype exhibited significant suppression.

In the late-pregnant control animals, the expressions of the α1A- and α1B-ARs did not differ from that in those exposed to AA, while the amount of α1D subtype mRNA was markedly increased in the myometrium of the rats subjected to inflammation (Fig. 4).

The expression of the β2-ARs at the mRNA level in samples from the nonpregnant animals did not reveal an AA-related difference, while on day 21 of pregnancy the mRNA level was markedly decreased in the myometrium of the treated rats (Fig. 4).
DISCUSSION

Uterine innervation has a unique plasticity governed by the endocrine milieu (26). During pregnancy, the uterus undergoes profound remodeling, involving gradual degeneration of the nerve fibers supplying both the myometrium and its vasculature. This pregnancy-induced denervation affects all types of nerves, i.e. adrenergic, cholinergic and peptidergic, but it is generally accepted that denervation of the adrenergic fibers is of much higher physiological importance than that of the others (5, 27-29). By term, the uterus can be regarded as a denervated organ which slowly becomes reinnervated after delivery, but the level of innervation never reaches that in the virgo state (5). The exact reason for the denervation has not been fully elucidated, but it is speculated that loss of the uterine nerves can contribute substantially to the functional isolation of the fetoplacental unit and therefore provide protection against α-AR-mediated vasoconstriction and myometrial contractions (30). Degeneration of the adrenergic fibers therefore plays a role in the prevention of these potentially harmful consequences (31). The present set of experiments revealed that experimental generalized inflammation can interact substantially with this physiological degenerational procedure, resulting in altered functions at both the pre- and the postsynaptic sites of adrenergic transmission. In the adrenergic part, the uptake and stimulated release of noradrenaline were decreased in the nonpregnant state, but not at term. This is in line with our previous result indicating that by term the functional denervation is complete, and therefore no further decrease is possible (32). Indeed, the stimulated transmitter release from the inflammation-exposed rats was not significantly less than that from the control animals and the uptake capacity detected at term can be regarded as the "background capacity".

Fig. 4. Adrenoceptor mRNA levels in myometrial samples from control (■) and inflammation-exposed (□) rats. Upper row: α₁A-AR subtypes in nonpregnant rats; middle row: α₁B-AR subtypes in late-pregnant rats; lower row: β₂-ARs in nonpregnant and late-pregnant rats. Data are means±S.E.M. (n=4). * and **: p<0.05 and p<0.01 as compared to the control values, respectively.
As the inhibitions of \( [^3H] \)noradrenaline release and tissue uptake are similarly pronounced in the virgo state and during pregnancy, this can be considered to be a general, rather than a gestation-specific phenomenon.

It is speculated that the deterioration of these presynaptic functions of the adrenergic transmission is a manifestation of the RA-induced neuropathy, including multiple mononeuropathies, sensorimotor neuropathy, entrapment neuropathy and vasculitis (2). These clinical pictures of peripheral nerve involvement are usually confined to patients with an aggressive form of RA or with signs of vasculitis (33). Our results lead us to suggest that the myometrium is at least as sensitive to this kind of functional deterioration as other tissues.

The role of the adrenergic system in the development of RA has been considered several times (34). In the early phase of experimental joint inflammation, \( \beta \)-ARs seem to predominate: the severity of AA was increased by \( \beta \)-blockade, and decreased by terbutaline treatment (35). In the later stages of inflammation, the \( \alpha \)-ARs gain a more prominent role. The \( \alpha_2 \)-ARs are partly responsible for connecting the sympathetic system to the nociceptive sensory system in chronic hyperalgesic rats (36). Another study found up-regulated \( \alpha_2 \)-ARs on leukocytes from juvenile chronic RA patients (37). During the progression of the disease, a \( \beta \)-to-\( \alpha \) adrenergic shift has been suggested as an overall description of the importance of the special receptors. In a more general interpretation, during inflammatory joint diseases the \( \beta \)-ARs undergo desensitization, while the \( \alpha \)-ARs are up-regulated, which can contribute to maintenance of the disorder (34). An anti-inflammatory effect of adrenaline mediated through \( \beta_1 \)-ARs has been reported previously (38).

Our results demonstrate that AA fundamentally alters the reproductive functions in both nonpregnant and pregnant rats. In pregnancies of rats complicated with joint disease, the \( \alpha \)- and \( \beta \)-AR functions displayed pronounced changes in the neuronal functions investigated by EFS.

These are the first reported data that these characteristic changes can be detected in the uterus, or, more exactly, that inflammation may modify the pregnancy-induced physiological changes in the myometrial adrenergic status. The exact mechanism of these modulatory effects remains enigmatic, but the experimental arthritis-induced increase in the cytokine level (\( e.g. \) IL-1\( \beta \) and IL-6) has been reported to decrease the \( \beta \)-adrenergic responsiveness, perhaps by heterologous desensitization of the \( \beta_2 \)-ARs in the smooth muscle (39-42). We detected a similar decrease in \( \beta_2 \)-AR expression in the late-pregnant myometrium, which resulted in a decreased relaxant effect of terbutaline. Besides this subtype, \( \beta_1 \)-AR is also expressed in the human myometrium and contributes to the overall relaxation induced by adrenaline (43).

As concerns the interplay of the expression of the \( \alpha_1 \)-ARs and the effect mediated through them, a more complex picture was obtained. In the nonpregnant state, inflammation has no influence on the contractile effect of noradrenaline, which is believed to be a result of balanced changes in the \( \alpha_1 \)- and \( \alpha_2 \)-ARs. At term, only the \( \alpha_1 \)-AR density responds to AA and its up-regulation is in accord with the increased contractile effect of noradrenaline without change in the EC\(_{50}\) value. It was previously reported that the \( \alpha_2 \)-ARs play a crucial role in the regulation of uterine motor activity in late pregnancy (44).

Overall, AA substantially modifies the contractility response of uterine rings to exogenous sympathomimetics, with an overall effect of an increase in motor activity as a result of decreased \( \beta \)- and increased \( \alpha \)-mediated relaxation and contraction, respectively. Pregnant patients with RA are at higher risk of preterm delivery, for which the exact pathomechanism is poorly elucidated, but similar changes in adrenergic receptor status cannot be excluded. At any rate, early contractions predispose these patients to tocolytic therapy, which means in practice treatment with \( \beta \)-mimetics. \( \alpha \)-AR antagonists have been suggested as alternative uterus relaxants with a more beneficial tolerability profile. Such agents have been advocated previously on the basis of animal experiments, but clinical data are not yet available (22). If similar modulations in AR status are operative in humans, \( i.e. \) inflammation-complicated pregnancies, the blockade of \( \alpha \)-ARs could be highly advocated over the use of traditional \( \beta \)-mimetics.

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