Inhibition of the tocolytic activity of atrial natriuretic factor by progesterone and potentiation by progesterone receptor antagonist RU486 in rats

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- 1 The influence of progesterone on the activity of atrial natriuretic factor (ANF) on rat myometrial motor activity was determined *in vitro*.
- 2 ANF inhibited the tension development by myometrium from cycling or oestrogen-treated rats in a dose-dependent manner; maximal inhibition was 100%.
- 3 Injections of progesterone into rats inhibited the tocolytic activity of ANF in a dose- and time-dependent manner. The tocolytic effects of ANF were completely abolished by 3 daily injections of 1 mg kg⁻¹ progesterone.
- 4 Pregnancy-related increase in plasma progesterone was accompanied by a corresponding decrease in the tocolytic effects of ANF; myometria from gestational day 10 to 21 were completely refractory and those from earlier gestational age and immediate postpartum were responsive to ANF to varying degrees.
- 5 Treatment of pregnant rats with the progesterone antagonist, RU486, caused abortions and vaginal bleeding, decreased plasma progesterone concentrations and restored the tocolytic activity of ANF. Tocolytic activity of ANF on virgin rat myometria was potentiated by RU486.
- 6 Progesterone also inhibited the effects of ANF on myometria from ovariectomized rats.
- 7 Tocolytic activity of isoprenaline was not modified by progesterone, pregnancy, RU486 or ovariectomy.
- 8 It is concluded that progesterone antagonizes myometrial effects of ANF by an oestrogen-independent mechanism and the pregnancy-induced refractoriness to the tocolytic effects of ANF is caused by progesterone.

Keywords: Progesterone; atrial natriuretic factor; RU486; plasma progesterone in pregnancy; tocolytic activity of atrial natriuretic factor; progesterone-atrial natriuretic factor interactions; ovariectomy

Introduction

Since the demonstration of the natriuretic and hypotensive activities of atrial extracts (deBold *et al.*, 1981), atrial natriuretic factor (ANF) has been shown to produce various biological effects (Brenner *et al.*, 1990). Homeostasis of ANF during pregnancy and its effects on female organs have also been investigated. ANF inhibits myometrial motor activity (Bek *et al.*, 1988; Potvin & Varma, 1990) and oocyte maturation (Tornell *et al.*, 1990); it increases placental blood flow (Chemtob *et al.*, 1989) and does not cross the placenta (Mulay & Varma, 1989). Receptors for ANF are present in placenta blood vessels (McQueen *et al.*, 1990) and tissues (Sen, 1986; Hatjis & Grogan, 1988; 1989), rat mammary glands (Pelletier, 1988) and myometrium (Potvin & Varma, 1991). Plasma concentrations of ANF increase during pregnancy and parturition in man (Otsuki *et al.*, 1987; Rutherford *et al.*, 1987; Steegers *et al.*, 1987; Thomsen *et al.*, 1987; Elias *et al.*, 1988; Hatjis *et al.*, 1989; Miyamoto *et al.*, 1989) and animals (Cheung *et al.*, 1987; Castro *et al.*, 1989; Olsson *et al.*, 1989). Vascular (St-Louis *et al.*, 1988; Chemtob *et al.*, 1989) and renal (Kristensen *et al.*, 1986) effects of ANF and placental vascular ANF receptors (McQueen *et al.*, 1990) are modified by pregnancy and pregnancy hypertension.

We recently showed that ANF inhibited the motor activity and increased guanylate cyclase activity and guanosine 3':5'-cyclic monophosphate levels of myometria from virgin rats; these effects of ANF were abolished or greatly attenuated by progesterone treatment and pregnancy (Potvin & Varma, 1990). On the basis of these results, we suggested that pregnancy-induced refractoriness to the tocolytic effects of

ANF might be caused by an increase in endogenous progesterone levels. The purpose of this study was to examine in greater detail the possible causal relationship between plasma progesterone during pregnancy and the inhibition of the tocolytic effects of ANF.

Methods

Animals and treatments

Rats were used in this study following the approval of the protocol by the McGill University Animal Care Committee.

Adult virgin Sprague-Dawley female rats (220–225 g) were purchased from Charles River, St. Constant, Quebec, Canada and maintained on Purina rat diet and tap water *ad libitum*. Animals were housed at 22–25°C, 50–70% humidity and a 12 h light (07 h 00 min–19 h 00 min) and 12 h dark (19 h 00 min–07 h 00 min) schedule. Presence of sperms in vaginal washing after overnight cohabitation with males denoted day zero of pregnancy. Bilateral ovariectomy was performed two weeks before experiments under ether anaesthesia. The stage of oestrous cycle was determined by microscopic examination of vaginal smears. Oestrogen (17 β -oestradiol) and progesterone were dissolved in peanut oil and injected intraperitoneally. The dose of oestrogen was 1 mg kg⁻¹ day⁻¹ for 2 days and similar to that used previously by us (Potvin & Varma, 1990) and others (Bek *et al.*, 1988). Doses of progesterone were 0.25, 0.5, 1.0 and 2.0 mg kg⁻¹ day⁻¹ for 3 days or 2 mg kg⁻¹ for 1, 2 or 3 days. Control rats were treated with peanut oil. A group of virgin and 17-day pregnant rats were treated orally once a day for two days with 10 mg kg⁻¹ RU486 suspended in 10% carboxymethyl cellulose (volume 1 ml kg⁻¹); controls for this

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group of studies were treated with 10% carboxymethyl cellulose. Drugs were administered between 09 h 00 min–10 h 00 min and myometrial activity determined 24 h after the last injection.

Determination of tocolytic activity in vitro

Rats were decapitated and uterine horns removed and set up in 50 ml tissue baths for recording isometric myometrial contractions on a Grass polygraph exactly as previously described (Potvin & Varma, 1990). Cumulative tocolytic concentration-response curves to ANF and isoprenaline were determined on separate strips from the same uterus. Myometrial tension development was quantitated by integration as described by Granger *et al.* (1985) and previously used by us (Potvin & Varma, 1990). Complete suppression of tension development was treated as 100% inhibition. Tocolytic potencies of ANF and isoprenaline were expressed as the $-\log$ molar concentration of drugs causing 50% inhibition in tension development ($-\log M EC_{50}$) and calculated from the regression line of the probit of percent effect versus $-\log M$ drug concentration for each experiment (Granger *et al.*, 1985). In cases where the maximal tocolytic effect was less than 100% but greater than 40%, $-\log M EC_{50}$ was calculated by extrapolation. EC_{50} was not calculated if the maximal tocolytic activity of ANF was less than 40%.

Plasma progesterone assays

In order to determine plasma progesterone profile during pregnancy, 0.3 ml blood was serially collected from the tail artery under brief periods (~ 2 min) of ether anaesthesia (Varma & Ramakrishnan, 1985) from 5 pregnant rats on days 0, 4, 7, 10, 14, 17, 19, 20 and 21 of gestation and within 12–24 h *postpartum*. Blood was collected from carboxymethyl cellulose-treated controls and RU486-treated animals just prior to killing them for the removal of uterine tissues. Plasma was separated by centrifugation (5000 r.p.m. for 2 min).

Plasma progesterone was measured by radioimmunoassay with highly specific antibodies as previously described (Mulay *et al.*, 1982). Approximately 800 c.p.m. of the tracer was added to the plasma and then extracted with methylene chloride; the extract was dissolved in phosphate-saline buffer. An aliquot was counted to estimate recovery and the remaining extract was incubated with approximately 6000–8000 c.p.m. of the tracer and the antibody for 2 h at 4°C. Separation of bound from free hormone was by adsorption onto dextran-coated charcoal. Intra- and interassay coefficients of variance were 8.5% and 8.1%, respectively. Each assay was done in duplicate and corrected for recovery.

Drugs and chemicals

Progesterone antagonist RU486 (17 β -hydroxy-11 β -(4-dimethylaminophenyl)-17 α -(prop-1-ynyl)estra-4,9-dien-3-one) was a gift from Roussel UCLAF, France. The following agents were purchased: synthetic rat atrial natriuretic factor (Hukabel, Montreal, Quebec, Canada), progesterone, oestrogen (17 β -oestradiol) and isoprenaline (Sigma Chemical, St. Louis, Missouri, U.S.A.), antiprogesterone antibodies (Endocrine Science, Tarzana, California, U.S.A.), [3H]-progesterone (100 Ci mmol $^{-1}$, Amersham, Oakville, Ontario, Canada), all other chemicals (BDH, Montreal, Quebec, Canada).

Statistics

Two means were compared by Student's *t* test; *F* values (variance ratios) did not differ at 5% probability. Multiple means were compared by one-way analysis of variance followed by comparison of each pair in the group (Bonferroni). A probability of less than 0.05 was assumed to denote a signifi-

cant difference. Throughout this paper, means $\pm$ s.e.mean are presented.

Results

Effects of atrial natriuretic factor on myometria from cycling rats

ANF inhibited the spontaneous motor activity of the uterus from cycling rats regardless of the stage of the oestrous cycle (Figure 1). However, the tocolytic potency of ANF was significantly lower on myometria from rats in metoestrus and dioestrus than on tissues from oestrogen-treated rats or rats in oestrus and pro-oestrus (Table 1).

Effect of progesterone on the tocolytic activities of atrial natriuretic factor

Treatment with progesterone inhibited the tocolytic activity of ANF in a dose-dependent (Figure 2a) and time-dependent (Figure 2b) manner. The tocolytic effect of ANF was completely abolished by 3 daily doses of 1 mg kg $^{-1}$ progesterone. However, ANF exerted some tocolytic effect when rats were treated with even a higher dose of progesterone (2 mg kg $^{-1}$) only for 1 or 2 days (Figure 2b, Table 2).

Effect of RU486 on the tocolytic effects of atrial natriuretic factor

Treatment of virgin cycling rats with RU486 significantly ($P < 0.05$) increased the tocolytic potency of ANF (Figure 3a) relative to that on myometria from vehicle-treated as well as oestrogen-treated animals (Table 3).

Administration of RU486 to 4 pregnant rats on days 17 and 18 of gestation caused expulsion of all foetuses in 2 rats and 6 of the 13 foetuses in 1 rat by day 19. The fourth rat retained all its foetuses but vaginal bleeding was noted. The mean plasma concentration of progesterone was significantly reduced relative to that on the corresponding day in vehicle-treated pregnant rats (Table 3). ANF was completely ineffective in vehicle-treated rats (Figure 3b, 4a). On the other hand, ANF caused 100% inhibition of the spontaneous motor activ-

Figure 1 Tocolytic concentration-response curves to atrial natriuretic factor on myometrial strips from rats in oestrus (○), pro-oestrus (△), metoestrus (●) and dioestrus (▲). Data are means of 4 experiments; vertical lines show s.e.mean.

Table 1 Tocolytic potency and effectiveness of atrial natriuretic factor on myometrial strips from oestrogen-treated, ovariectomized and cycling rats

Animals	n	Tocolytic ($-\log M EC_{50}$)	Tocolytic effectiveness (%)
Oestrogen-treated	19	8.01 ± 0.11^a	100 ^a
Pro-oestrus	4	7.86 ± 0.24^a	100 ^a
Oestrus	4	7.98 ± 0.14^a	100 ^a
Metoestrus	4	7.46 ± 0.11	80 ± 11
Dioestrus	4	7.49 ± 0.17	83 ± 16
Ovariectomized	11	7.22 ± 0.12	50 ± 8
Ovariectomized plus progesterone-treated	4	>6	0

Oestrogen ($1 \text{ mg kg}^{-1} \text{ day}^{-1} \times 2$) and progesterone ($2 \text{ mg kg}^{-1} \text{ day}^{-1} \times 3$) were injected intraperitoneally and ovariectomy was performed 2 weeks before experiments. Data are means $\pm$ s.e.mean.

^a Different ($P < 0.01$) from all values in the same column without the superscript but not from each other.

ity of myometrium from pregnant rats treated with RU486 (Figures 3b and 4b).

Effect of progesterone on the tocolytic activity of atrial natriuretic factor in ovariectomized rats

Relative to responses of myometria from cycling and oestrogen-treated virgin rats, ovariectomy caused a significant ($P < 0.05$) reduction in both the tocolytic effectiveness and potency of ANF. Treatment of ovariectomized rats with pro-

gesterone ($2 \text{ mg kg}^{-1} \times 3$) abolished the tocolytic activity of ANF (Table 1).

Plasma progesterone and tocolytic activity of atrial natriuretic factor

Injection of RU486 into virgin rats caused a small but insignificant increase in plasma progesterone. In contrast, administration of RU486 into pregnant rats caused a significant ($P < 0.001$) decrease in plasma progesterone (Table 3).

Plasma progesterone in pregnant rats progressively increased with gestational duration reaching a peak on day 14

Figure 2 Tocolytic concentration-response curves to atrial natriuretic factor on myometrial strips from virgin rats treated intraperitoneally with progesterone at different dose levels (a) and time-periods (b). (a) Vehicle (peanut-oil)-treated controls (●); treated daily for 3 days with progesterone at dose levels of 0.25 mg kg^{-1} (▲), 0.5 mg kg^{-1} (■), 1 mg kg^{-1} (○) and 2 mg kg^{-1} (△). (b) Vehicle (peanut-oil)-treated controls (●); treated with 2 mg kg^{-1} progesterone for 1 day (▲), 2 days (■) and 3 days (○). Injections were made intraperitoneally between 9 h 00 min–10 h 00 min and experiments done 24 h after the last injection. Data are means $\pm$ s.e.mean of 4–5 experiments.

Figure 3 Effects of the antiprogestosterone agent RU486 ($10 \text{ mg kg}^{-1} \text{ day}^{-1} \times 2$) on the tocolytic concentration-response curves to atrial natriuretic factor on myometrial strips from (a) virgin and (b) pregnant rats. RU486 (●) and the vehicle, 10% carboxymethyl cellulose (○) were administered orally. Pregnant rats were treated on days 17 and 18 and experiments were done on day 19 of gestation. Data are means $\pm$ s.e.mean of 4 experiments; s.e.mean shown by vertical lines.

Table 2 Effects of progesterone injections into virgin rats on the tocolytic activity of atrial natriuretic factor *in vitro*

Progesterone		Tocolytic ($-\log M EC_{50}$)	Tocolytic effectiveness (%)
Dose (mg kg^{-1})	Duration (days)		
0 (peanut oil)	3	7.69 ± 0.13^a	100 ^a
0.25	3	7.38 ± 0.16	91 ± 3
0.50	3	6.67 ± 0.11	47 ± 8
1.00	3	>6	0
2.00	3	>6	0
2.00	1	7.07 ± 0.18	76 ± 8
2.00	2	>6	22 ± 9
2.00	3	>6	0

Progesterone was dissolved in peanut oil and injected intraperitoneally between 9 h 00 min–10 h 00 min and experiments done 24 h after the last injection.

Data are means $\pm$ s.e.mean of 4 experiments.

^a Different ($P < 0.01$) from all other values in the same column.

Figure 4 An example of the effect of atrial natriuretic factor (ANF) on the spontaneous motor activity of myometrial strips from (a) vehicle (10% carboxymethyl cellulose)-treated and (b) anti-progesterone agent RU486-treated pregnant rats. Vehicle or RU486 were administered orally on days 17 and 18 and the experiment done on day 19 of gestation. Concentrations of ANF (nM) are indicated at the arrows.

and decreased to below day 0 levels within 24 h *postpartum* (Figure 5). The increase in plasma progesterone was associated with a progressive decrease in the tocolytic activity of ANF (Figure 5). Myometria from day 10 to day 21 pregnant rats were totally refractory to ANF. A significant tocolytic

Figure 5 Relationship between plasma progesterone concentrations (●) and the tocolytic efficacy of atrial natriuretic factor, (ANF, ▲) in pregnant rats. Blood was collected serially from the same 5 rats on different days of gestation and 12–24 h *postpartum* (PP) for the plasma progesterone assays. Tocolytic concentration-response curves to ANF were determined on myometrial strips of 4–5 rats killed on different days of gestation and on days 1 and 2 *postpartum* (PP). Vertical lines show s.e.mean.

activity of ANF was observed on *postpartum* day 1. By *postpartum* day 2, the tocolytic effect of ANF was comparable to that found in cycling virgin rats (Figure 5).

Tocolytic activity of isoprenaline

The tocolytic potency ($-\log M EC_{50}$) and effectiveness (%) of isoprenaline on myometria from oestrogen-treated rats ($n = 13$) was 9.58 ± 0.11 and 100%, respectively, and was not significantly altered by the stage of oestrous cycle, pregnancy, ovariectomy, progesterone treatment and RU486 treatment (data not shown).

Discussion

In an earlier paper, we had suggested that the pregnancy-induced refractoriness to the tocolytic activity of atrial natriuretic factor (ANF) is mediated by progesterone (Potvin & Varma, 1990). The main objective of the present study was to test this hypothesis by examining the relationship between plasma progesterone and the tocolytic activity of ANF. We also argued that if our hypothesis is correct, progesterone antagonist RU 486 (Baulieu, 1989) should restore the tocolytic effects of ANF on myometrium from pregnant rats. The data of this study confirm all related observations described earlier (Potvin & Varma, 1990) and provide strong support for our hypothesis. On the other hand, the uterus is exposed to varying concentrations of oestrogen and progesterone under different physiological states in man (Danforth & Scott, 1986) and rats (Shaikh, 1971; Morishige *et al.*, 1973; Butcher *et al.*, 1974). Oestrogen and progesterone exert antagonistic effects on myometrial activity (Downing *et al.*, 1981) and on the tocolytic activity of ANF (Potvin & Varma, 1990). Thus endogenous oestrogen may mask by varying degree depending upon its plasma titre the influence of progesterone on the tocolytic activity of ANF. If so, progesterone dominance would be required for a full expression of its influence on the myometrial activity of ANF.

The sensitivity of the myometrium to ANF was greater during pro-oestrus and oestrus than during metoestrus and dioestrus (Table 1). Although oestrogen levels are highest during pro-oestrus (Shaikh, 1971; Butcher *et al.*, 1974), the highest progesterone levels are achieved just before oestrus (Butcher *et al.*, 1974). One would have expected a decreased response of the myometrium from rats in oestrus to ANF; the opposite, however, was the case. It is logical to assume that there is a lag period between the exposure of the uterus to these steroids and their influence on the tocolytic activity of ANF; indeed, this is suggested by our data that a single injection of a rather high dose of progesterone (2 mg kg^{-1}) caused far less inhibition of the effects of ANF than did a smaller dose of 1 mg kg^{-1} administered for three consecutive days (Table 2).

Notwithstanding the above, results of this study clearly show that an increase in plasma progesterone by exogenous administration (Figure 2) or increased endogenous production

Table 3 Effect of antiprogesterone agent RU486 on plasma progesterone levels and the tocolytic activity of atrial natriuretic factor on myometrial strips from virgin and day 19 pregnant rats

Animals	Treatment	Plasma progesterone (nmol l ⁻¹)	Tocolytic ($-\log M EC_{50}$)	Tocolytic effectiveness (%)
Virgin	Oestrogen	174 ± 77	8.01 ± 0.09	100
Virgin	Vehicle	64 ± 13	7.61 ± 0.15	100
Virgin	RU486	204 ± 96	8.29 ± 0.10^a	100
Pregnant	Vehicle	456 ± 66	> 6	0
Pregnant	RU486	71 ± 30^a	7.79 ± 0.15^a	100

Vehicle (1 ml kg^{-1} of 10% carboxymethyl cellulose) and RU486 (10 mg kg^{-1}) were administered orally once a day for 2 days, on days 17 and 18 of gestation in the case of pregnant rats and experiments were done 24 h after the second treatment. Oestrogen was administered at a dose of $1 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 2 days.

Data are means $\pm$ s.e.mean of 4–5 experiments except for the tocolytic activity in oestrogen-treated group, which is derived from 19 experiments.

^a Different ($P < 0.01$) from the immediate top value.

such as during pregnancy (Figure 5), results in a marked decrease in the tocolytic activity of ANF. The profile of plasma progesterone as determined by serial blood collections from the same animals from day 0 to postpartum period are very similar to values described by other workers (Morishige *et al.*, 1973) and the increase in plasma progesterone is temporally related to a decrease in the tocolytic effects of ANF. Likewise a rapid decrease in plasma progesterone just preceding the parturition corresponds with a recovery of the tocolytic activity of ANF in the *postpartum* period.

A more direct evidence that pregnancy-induced refractoriness to the tocolytic activity of ANF is caused by progesterone comes from studies using the progesterone receptor antagonist RU486 (Baulieu, 1989). Thus the myometrium totally refractory to ANF on day 19 of gestation was fully responsive to it after the administration of RU486. Although the administration of RU486 caused a decrease in plasma progesterone due to obvious or impending abortion, the restoration of the tocolytic effects of ANF appears to be due to a blockade of progesterone receptors by RU486 rather than a consequence of abortion. This inference is supported by two observations. Firstly, the myometrium of one rat which had not aborted was responsive to ANF and second, RU486 potentiated the tocolytic effects of ANF in virgin rats without significantly altering plasma progesterone levels.

The exact mechanism by which progesterone inhibits the tocolytic effects of ANF is not clear from our studies. Oestro-

gen and progesterone appear to exert antagonistic effects on myometrial activity (Downing *et al.*, 1981). It may therefore be inferred that progesterone inhibits the activity of ANF by antagonizing oestrogen. However, our results suggest that this is not an absolute requirement for progesterone-induced refractoriness to the effects of ANF since this influence of progesterone was also observed in ovariectomized rats. This observation along with data that the effects of progesterone could be abolished by RU486 suggest that the inhibition of the tocolytic effects of ANF by progesterone involves an action of the steroid on its receptors leading to induction of certain factors, possibly proteins, which cause a decrease in myometrial ANF receptors (Potvin & Varma, 1991) and in turn a decrease in the tocolytic effects of ANF.

In summary, data of the present study strongly support the hypothesis that the pregnancy-induced inhibition of the tocolytic activity of ANF results from the exposure of the uterus to relatively high concentrations of progesterone. Plasma concentrations of ANF increase during pregnancy. Whereas this increase in plasma ANF during pregnancy might be a physiological response to fluid expansion, the decrease in the tocolytic activity of ANF might be desirable for foetal development and parturition.

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