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Potentiation of the hypotensive effect of adrenomedullin in pregnant rats

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Abstract

The hypotensive effect of adrenomedullin, a potent vasodilator peptide, was examined in conscious pregnant (6, 13 and 20 days of pregnancy) and non-pregnant rats. The intravenous administration of adrenomedullin (0.01–3.0 nmol/kg) produced a dose-dependent depressor response in pregnant and non-pregnant rats. At low doses (0.01–0.1 nmol/kg), the maximum decrease in blood pressure was significantly higher in pregnant rats (20 days pregnant) than in non-pregnant rats. At high doses, no significant difference was observed between the two groups. Furthermore, the administration of adrenomedullin did not significantly affect the basal mean blood pressure (MBP) at any dose when compared to the non-pregnant group at 6 and 13 days of pregnancy. In the ovariectomized rats, the depressor responses in 17β-estradiol-treated, progesterone-treated and 17β-estradiol + progesterone-treated rats were not significantly different from that in the control rats, suggesting that the augmented effect on the depressor response to adrenomedullin in pregnant rats may not be due to hormonal changes during pregnancy. The adrenomedullin receptor mRNA level of the descending thoracic aorta was significantly higher in the late-pregnancy rats (20 days of pregnancy). However, the levels did not show any difference between the early-pregnant rats (6 and 13 days of pregnancy) and the non-pregnant rats. These findings suggested that the changes in the depressor response to adrenomedullin which occur at term in pregnant rats may be mediated by changes of adrenomedullin receptor gene expression rather than by sex hormones.

Keywords: Adrenomedullin; Pregnancy; Blood pressure; (Rat)

1. Introduction

Adrenomedullin, a potent vasorelaxant/hypotensive peptide with 52 amino acid residues, was recently isolated from human pheochromocytoma (Kitamura et al., 1993a). Subsequent studies have shown adrenomedullin immunoreactivity to be detectable in plasma (Nishikimi et al., 1994) and it is also widely distributed in lung, lung tumor, adrenal gland, heart atrium, kidney, and pancreas (Kitamura et al., 1993b). The expression of mRNA for adrenomedullin has been demonstrated in these tissues (Martínez et al., 1995, 1996). Endothelial cells are also known to produce adrenomedullin (Sugo et al., 1994). In addition, an increase of adrenomedullin concentration in the plasma of patients with essential hypertension was observed when compared with the normotensive controls (Kitamura et al., 1994). Such evidence implies that adrenomedullin plays a role in the regulation of blood pressure and vascular homeostasis.

During pregnancy, a number of physiological changes occur in the maternal circulation to accommodate the growing fetus. These changes usually include an increase in cardiac output and a decrease in arterial blood pressure and total peripheral resistance (Poston et al., 1995; Baylis et al., 1996; Slangen et al., 1996). The attenuation of pressor responsiveness to several administered vasoconstrictors is a constant feature of normal pregnancy in humans (Gant et al., 1973) and other species, such as rats (Paller, 1984; Conrad and Colpoys, 1986). However, the mechanism of this physiological adaptation remains uncertain. Because the plasma levels of 17β-estradiol and progesterone increase markedly during pregnancy (Cunningham et al., 1989), several investigators reported that administration of 17β-estradiol (Tamai et al., 1984; Yoshimura et al., 1984) and/or progesterone (Hettiaratchi

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and Pickford, 1968; McLaughlin et al., 1985) to various animal species produces attenuation of the pressor responsiveness or vascular reactivity to several vasoconstrictors. On the other hand, other studies did not find estradiol and/or progesterone to have such an effect (Novak and Kaufman, 1991).

Recent studies have shown an increased plasma adrenomedullin concentration in pregnant women (Di Iorio et al., 1997). In addition, high intense staining for immunoreactive adrenomedullin was also found in the placenta and fetal membrane (Marinoni et al., 1998). More recently, we demonstrated that adrenomedullin attenuates the nitric oxide (NO) synthase inhibitor-induced hypertension in pregnant rats, but not in non-pregnant rats (Makino et al., 1999). These findings suggest that adrenomedullin thus plays some role in pregnancy. However, the role that adrenomedullin plays in mediating the hemodynamic changes in pregnancy is still unclear.

Therefore, the present study was designed to examine the depressor response to adrenomedullin in pregnant and non-pregnant rats in various gestational stages. We also examined the effects of hormones on vascular reactivity to adrenomedullin in rats.

2. Materials and methods

2.1. Animals

Pregnant and non-pregnant rats (Wistar strain), 250–300 g, obtained from the Kyudo Animal Laboratory (Kumamoto, Japan) were maintained in an animal room with a 12-h light–dark cycle (0800–2000 h). The rats were divided into four groups, (a) non-pregnant; (b) pregnant in early gestation (6 days pregnant) ; (c) pregnant in mid-gestation (13 days pregnant); and (d) pregnant in late gestation (20 days pregnant). The day that sperm cells were first seen in the vaginal lavage was considered to be day 0 of pregnancy. All animals were given free access to food and water. The guidelines approved by the animal research committee of Fukuoka University for the care and use of experimental animals were all closely observed.

2.2. Blood pressure measurement

For the direct recording of arterial blood pressure, the animals were anesthetized with ether. An arterial catheter, composed of polyethylene tubing (PE-10 and PE-20), was chronically implanted into the abdominal aorta through the left femoral artery, as described previously (Kawasaki et al., 1987; Shibata et al., 1993). At the same time, a catheter for the intravenous administration of adrenomedullin was implanted chronically into the inferior vena cava through the left femoral vein. The remainder was passed beneath the skin to emerge at the back of the neck and then was plugged with a stainless steel stopper. The blood pressure was measured in an unrestrained, conscious state. The animals were injected with six graded doses of adrenomedullin (0.01–3.0 nmol/kg) in a random fashion and changes in mean blood pressure (MBP) and heart rate were recorded. Time was allowed for the blood pressure to return to its baseline (40–50 min) after injection. The maximal changes in MBP and heart rate caused by adrenomedullin were examined. To provide a description of both the duration and magnitude of the cardiovascular response, the area under the curve (AUC) was calculated for the 30-min period immediately after peptide injection.

2.3. Treatment of ovariectomized rats with estradiol or progesterone

In female virgin rats (4 weeks old), bilateral oophorectomy was performed by laparotomy under anesthesia with light ether. They were allowed to recover in the animal center for 14 days. The animals were next divided into four treatment groups and received a daily injection (0.1% body weight, s.c.) of corn oil (control group), 17β-estradiol (0.1 mg kg^{-1} day^{-1}), progesterone (2 mg kg^{-1} day^{-1}) or a mixture of both estradiol (0.1 mg kg^{-1} day^{-1}) and progesterone (2 mg kg^{-1} day^{-1}) for a period of 21 days. Estradiol and progesterone were obtained from Sigma (St Louis, MO, USA). The doses of each hormone were chosen according to a previous report (Nakamura et al., 1988). The hormone-treated rats were studied for vascular reactivity to adrenomedullin.

2.4. Northern blot analysis

Rats were killed with an overdose of sodium pentobarbital. The descending thoracic aorta was rapidly obtained on days 6, 13 and 20 of pregnancy.

Total RNA was extracted using guanidine isothiocyanate according to a method described previously (Makino et al., 1996). The RNA extract was quantified by measuring absorbance at 260 nm.

The Northern blot analysis was essentially the same as that described in our previous report (Shibata et al., 1997). The total RNA (10–30 μg) from the aorta was separated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane. The hybridization probe for adrenomedullin receptor mRNA was generated by the reverse transcriptase/polymerase chain reaction as previously reported (Cormier-Regard et al., 1998). The 471-bp fragment of adrenomedullin receptor cDNA was labeled by random primer labeling and used as a hybridization probe.

After hybridization, the blots were washed three times in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 15 min at 65°C. The filters were rehybridized with a radiolabeled G3PDH (glyceraldehyde 3-phosphate dehydrogenase) probe. The blots were exposed to X-ray film (Kodak, XAR-5) with an

intensifying screen at −80°C for various lengths of time and then were scanned with a laser densitometer (Personal densitometer SI, Molecular Dynamics, USA) to determine the individual band density. The mean densitometric units derived from the controls were standardized to a value of 1.00.

2.5. Nuclear run-off transcription assay

Nuclear run-off assays were carried out based on the procedure of White and LaGamma (1989). The descending thoracic aorta (20-days-pregnant rats and non-pregnant rats) was rapidly removed, immediately placed in 1 ml of homogenization buffer (10 mM Tris, pH 8.0, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.3 M sucrose, 0.15% Triton X-100) and allowed to swell for 10 min. Following gentle homogenization with a Teflon pestle, the homogenate was layered over 0.5 ml of centrifugation buffer (10 mM Tris, pH 8.0, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 M sucrose) and centrifuged. The nuclear pellet was resuspended in 100 μl of reaction mix (20 mM Tris, pH 7.9, 20% glycerol, 140 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/ml creatine phosphokinase, 8.5 mM phosphocreatine, 2 mM each ATP, CTP and GTP, 1 unit/μl RNasin and 250 μCi of [^{32}P]UTP. The tubes were incubated at 30°C for 30 min. After DNase I and proteinase K digestion, the reaction products were extracted with phenol/chloroform; thereafter, unincorpo-

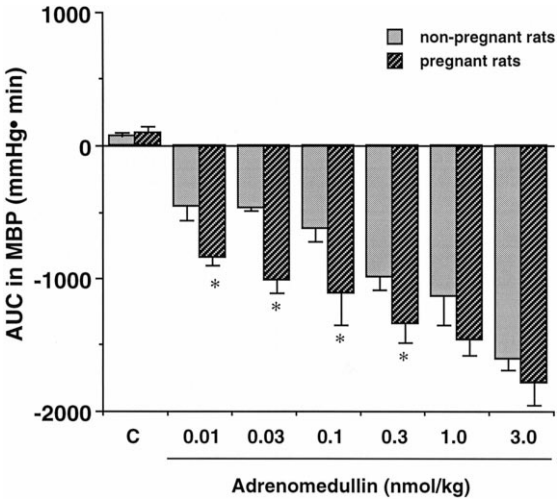

Fig. 2. Effects of adrenomedullin on MBP in late gestation in pregnant rats (day 20 of pregnancy) and non-pregnant rats. Data are presented as AUC (mean ± S.E.M.). $^*P < 0.05$ compared with the non-pregnant rats (Student's *t*-test) ($n = 5–6$). Filled bars, non-pregnant rats; striped bars, pregnant rats. C, saline. Further details in Fig. 1.

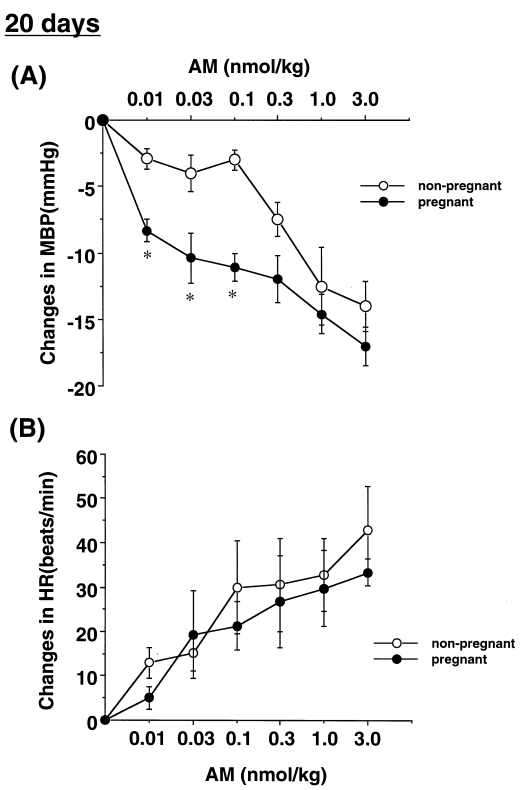

Fig. 1. Effects of adrenomedullin on the MBP (A) and heart rate (B) in late gestation in pregnant rats (day 20 of pregnancy) and non-pregnant rats. Adrenomedullin at 0.01–3.0 nmol/kg was injected intravenously in conscious rats. Each point represents the mean ± S.E.M. $^*P < 0.05$ compared with the non-pregnant rats (Student's *t*-test) ($n = 5–6$). (○) Non-pregnant rats. (●) Pregnant rats. AM, adrenomedullin; MBP: mean blood pressure; HR: heart rate.

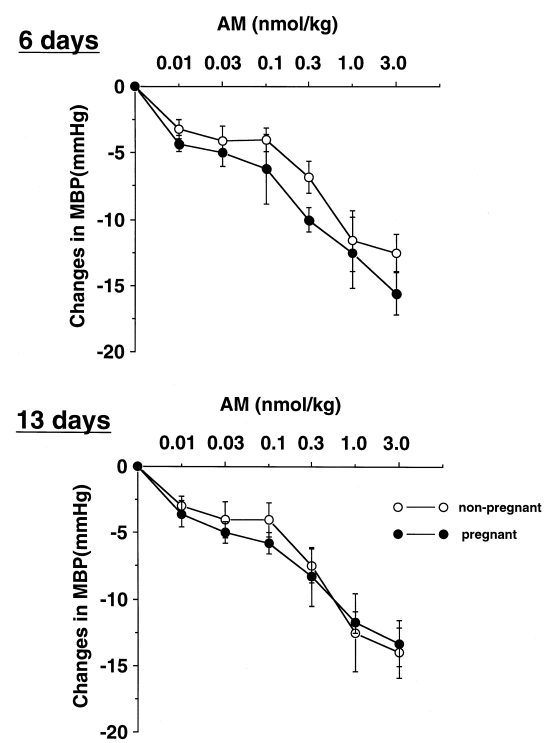

Fig. 3. Hypotensive effect of intravenous adrenomedullin on MBP in early (day 6 of pregnancy) and mid-gestation (day 13 of pregnancy) in pregnant rats. Each point represents the mean ± S.E.M. ($n = 4–6$) (○) Non-pregnant rats. (●) Pregnant rats. AM, adrenomedullin.

rated [^{32}P]UTP was removed by filtration. The radiolabeled RNA was hybridized at 42°C for 48 h with 5 μg of linearized pGEM plasmid and immobilized to a nylon membrane, containing rat adrenomedullin receptor or rat G3PDH fragments. The filters were washed for 30 min in 0.2× SSC (15 mM NaCl, 1.5 mM sodium citrate)/0.1% SDS at 65°C and placed in film cassettes for 2–4 days.

2.6. Transcript stability analysis

The stability of the adrenomedullin receptor mRNA was investigated in primary cultures of vascular smooth muscle cells of 20-day pregnant and non-pregnant rats. Rat vascular smooth muscle cells were isolated from the descending thoracic aorta by treatment with 0.3% collagenase and 0.05% elastase, and maintained in Dulbecco's modified eagle medium containing 20% fetal calf serum. The stability of the adrenomedullin receptor mRNA was examined by inhibiting new mRNA transcription with actinomycin D (5 μg/ml). The confluent vascular smooth muscle cells were incubated with actinomycin D. After various lengths of incubation, the total RNA was isolated from individual dishes and the disappearance of mRNA abundance was determined by Northern blotting.

2.7. Statistical analysis

All values were expressed as the means ± S.E.M. The data were compared by either Student's *t*-test or Dunnett's test.

3. Results

3.1. Effect of pregnancy on depressor response to adrenomedullin

The basal MBP before administration of adrenomedullin in 6, 13, 20 days of pregnancy and non-pregnant was 100.7 ± 1.4 mmHg ($n = 17$), 99.8 ± 1.3 mmHg ($n = 18$), 93.9 ± 2.0 mmHg ($n = 30$) and 107.9 ± 1.1 mmHg ($n = 30$), respectively. The basal levels of blood pressure were significantly lower in 20-days-pregnant rats than in the non-pregnant rats ($P < 0.05$), as previously reported (Castro et al., 1993).

As shown in Fig. 1A, the administration of adrenomedullin (0.01–3.0 nmol/kg) produced a dose-dependent depressor response in pregnant (20 days of pregnancy) and non-pregnant rats. The depressor response to adrenomedullin had a quick onset (10 s) and lasted for about 20 min.

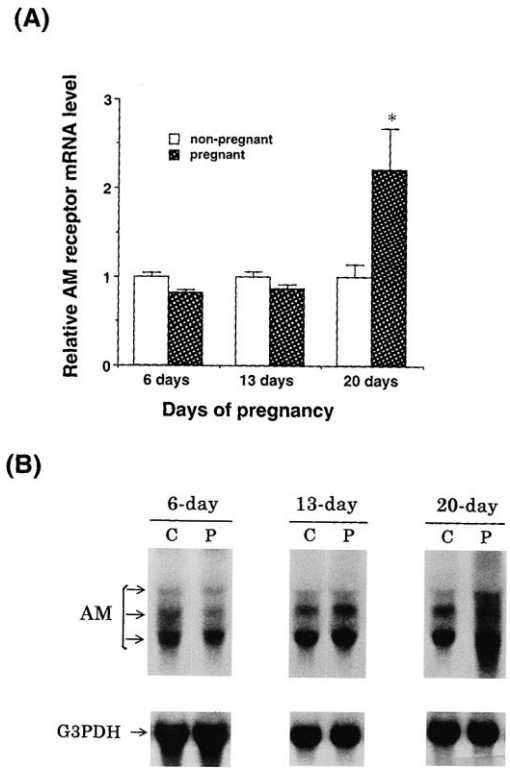

Fig. 4. Adrenomedullin receptor mRNA expression during pregnancy. (A) The relative amounts of adrenomedullin receptor mRNA in non-pregnant and pregnant rats. The mRNA level was determined by Northern blot analysis. The adrenomedullin receptor mRNA level in non-pregnant rats of each age was, respectively, set as 1.00 and compared with those of age-matched pregnant rats. Open columns, non-pregnant rats; hatched columns, pregnant rats. * $P < 0.05$, compared with age-matched non-pregnant rats (Student's *t*-test) ($n = 5–7$). (B) A representative autoradiogram of adrenomedullin receptor mRNA expression. C, control (non-pregnant rat). P, pregnant rat. G3PDH, glyceraldehyde 3-phosphate dehydrogenase. AM, adrenomedullin receptor.

Table 1
Hypotensive response (mmHg) to adrenomedullin in ovariectomized rats given estradiol or progesterone
Values represent the means ± S.E.M. AM, adrenomedullin. The drugs were injected subcutaneously for 21 days.

Treatment	AM (nmol/kg, i.v.)					
	0.01	0.03	0.1	0.3	1.0	3.0
Ovariectomized ($n = 9$)	−5.8 ± 2.0	−6.1 ± 1.5	−7.2 ± 1.8	−7.1 ± 1.9	−13.8 ± 0.9	−17.5 ± 1.5
17β-estradiol-treated ($n = 9$)	−6.3 ± 1.6	−8.7 ± 2.9	−8.1 ± 1.5	−10.0 ± 1.8	−14.3 ± 1.1	−18.0 ± 1.4
Progesterone-treated ($n = 9$)	−5.6 ± 1.8	−6.0 ± 1.0	−8.7 ± 0.9	−9.5 ± 1.0	−15.6 ± 2.0	−18.3 ± 1.7
17β-estradiol ($n = 8$) + progesterone-treated	−6.3 ± 1.4	−6.7 ± 1.1	−8.9 ± 1.8	−11.5 ± 2.0	−13.5 ± 0.6	−17.8 ± 0.6

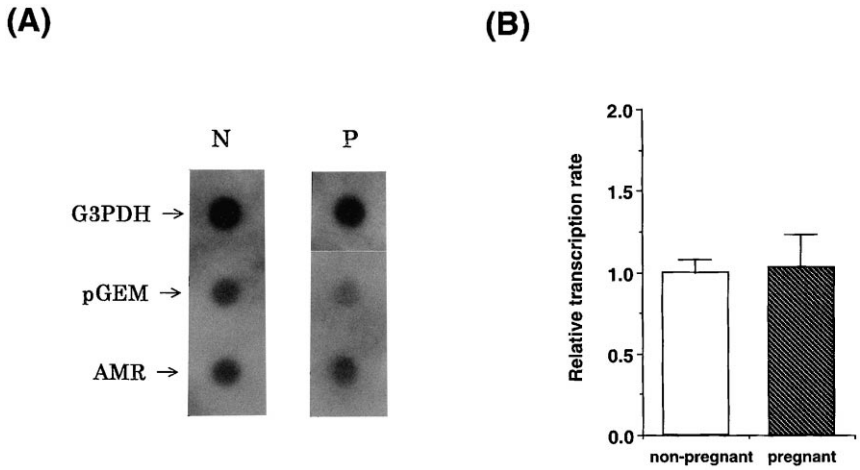

Fig. 5. Nuclear run-off assays of the transcriptional rate of the adrenomedullin receptor gene in pregnant (20 days of pregnancy) and non-pregnant rats. (A) A representative autoradiogram. (B) The relative transcription rate of the adrenomedullin receptor gene. Descending thoracic aortas were used. The radiolabeled RNA was hybridized with linearized pGEM vector alone (5 μg), pGEM containing rat adrenomedullin receptor cDNA (5 μg) or G3PDH (5 μg). The adrenomedullin receptor transcriptional rates are expressed relative to those of G3PDH gene after their background levels (pGEM vector alone) were reduced, and the relative values in non-pregnant rats are normalized to 1.00. The results shown are the means ± S.E.M. of three separate experiments. N, non-pregnant rats. P, pregnant rats. G3PDH, glyceraldehyde 3-phosphate dehydrogenase. AMR, adrenomedullin receptor.

However, the maximum decrease in pressure was significantly higher in pregnant rats than in non-pregnant rats at low doses (0.01–0.1 nmol/kg). As shown in Fig. 1B, adrenomedullin also induced an increased heart rate in both groups of animals, as previously reported (Parkes and May, 1997). However, as shown in Fig. 1B, the heart rate did not show any difference between both groups. As shown in Fig. 2, the results of the AUC analysis indicate that the depressor response in the 20 days pregnant rats was greater and longer-lasting than that in the non-pregnant rats at four doses (0.01–0.3 nmol/kg).

On the other hand, in animals in early and mid-gestation, as shown in Fig. 3, the administration of adrenomedullin did not significantly affect the MBP at any dose when compared to that of the non-pregnant group.

3.2. Effect of estradiol or progesterone on the depressor response to adrenomedullin in ovariectomized rats

The depressor responses to adrenomedullin in ovariectomized rats did not differ from those in non-pregnant rats, as indicated in Table 1.

As shown in Table 1, the depressor responses in 17β-estradiol-treated, progesterone-treated and 17β-estradiol + progesterone-treated rats were not significantly different from those in the ovariectomized control rats, though the depressor response in the progesterone-treated group did tend to increase.

3.3. Analysis of the adrenomedullin receptor mRNA level in the thoracic aorta from pregnant and non-pregnant rats

The bands of the expected 1.8-, 3.0-, and 5.0-kb size were seen in all groups. These sizes were consistent with those from a previous report (Kapas et al., 1995).

As shown in Fig. 4, the adrenomedullin receptor mRNA level of the descending thoracic aorta was significantly higher in pregnant rats (20 days of pregnancy) than that in aorta from non-pregnant rats. However, no significant difference was observed between the abundance of mRNA in non-pregnant and pregnant rats in the early and mid-gestation period.

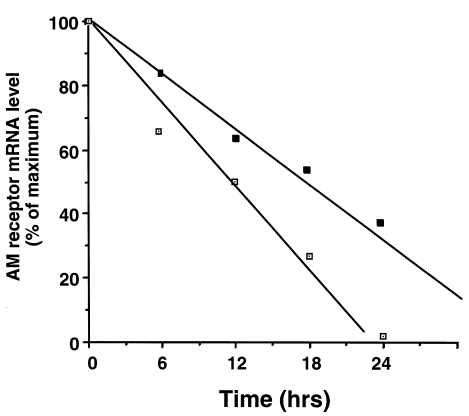

Fig. 6. Stability of adrenomedullin receptor mRNA. The stability of the adrenomedullin receptor mRNA was investigated in primary cultures of vascular smooth muscle cells of 20-days-pregnant and non-pregnant rats. Rat vascular smooth muscle cells were isolated from the descending thoracic aorta by treatment with 0.3% collagenase and 0.05% elastase, and maintained in Dulbecco's modified eagle medium containing 20% fetal calf serum. Stability of the adrenomedullin receptor mRNA was examined by inhibiting new mRNA transcription with actinomycin D (5 μg/ml). The confluent vascular smooth muscle cells were incubated with actinomycin D. After various times of incubation, total RNA was isolated from individual dishes and the disappearance of mRNA abundance was determined by Northern blotting. The data shown are the means of three separate experiments. Open square, non-pregnant rats; filled square, pregnant rats.

As shown in Fig. 5, the relative rate of the adrenomedullin receptor gene transcription was not significantly different between 20-days-pregnant and non-pregnant rats.

The stability of the adrenomedullin receptor mRNA was also examined by inhibiting new mRNA transcription with actinomycin D. As shown in Fig. 6, the half-life (17.8 ± 0.2 h) in pregnant rats (day 20 of pregnancy) was significantly longer than that in the non-pregnant rats (13.4 ± 0.3 h, $P < 0.05$).

4. Discussion

In the present study, we demonstrated for the first time that the decrease in blood pressure following adrenomedullin at low doses (0.01–0.3 nmol/kg) was significantly greater in pregnant rats (20 days of pregnancy) than that in non-pregnant rats. At 6 and 13 days of pregnancy, there were no significant differences in the change of MBP when compared to the non-pregnant rats. These findings suggest that the depressor response to adrenomedullin, especially at low doses, increased significantly in pregnant rats in late gestation in comparison to the non-pregnant rats.

During pregnancy, a number of physiological changes occur in the maternal circulation to accommodate the growing fetus. These changes usually include an increased cardiac output and a decreased MBP and total peripheral resistance (Poston et al., 1995; Baylis et al., 1996; Slangen et al., 1996).

In addition, it is generally agreed that pregnant animals exhibit a decreased pressor response to angiotensin II and norepinephrine (Gant et al., 1973; Paller, 1984; Conrad and Colpoys, 1986). However, the exact mechanism for the altered vascular sensitivity during pregnancy is still being debated.

As for the mechanism underlying the altered sensitivity to adrenomedullin in late gestation in the present study, a hormonal effect is a possibility. In fact, a previous report demonstrated that the decreased pressor response to angiotensin II in pregnant rats is mainly mediated by progesterone (Nakamura et al., 1988). We thus studied the effect of these hormones on vascular reactivity to adrenomedullin in pregnant rats. In our study, treatment of rats with 17β-estradiol, progesterone or 17β-estradiol + progesterone had no effect on the pressor response to adrenomedullin in ovariectomized rats. Minamino et al. (1995) reported that 17β-estradiol and progesterone did not affect adrenomedullin production in cultured rat vascular smooth muscles cells. These findings suggest that these hormones did not have any effect on adrenomedullin secretion. Based on such evidence, we are unable to support the hypothesis that these hormones mediate the potentiation of depressor responsiveness observed during late gestation.

Another possibility for potentiation of depressor responsiveness during late gestation is that of changes in cardiovascular reflex system. A number of studies exist in which the cardiovascular reflex mechanisms were examined in pregnancy. Cumbee et al. (1986) suggested that the baroreflex-mediated increases in efferent renal sympathetic nerve activity may be attenuated in pregnancy. In fact, previous reports suggested that pregnant animals show an attenuated ability to increase the sympathetic outflow in response to a hypotensive challenge, such as captopril treatment (Crandall and Heesch, 1990). As a result, the authors demonstrated that the blood pressure decreased to a greater extent in the pregnant rats than in the non-pregnant rats after captopril treatment. It is, therefore, also possible that adrenomedullin exerts a potentiated hypotensive response based on changes in the baroreflex in pregnant rats. On the other hand, the hypotensive effect of atrial natriuretic peptide, which is a potent vasodilator, was blunted in pregnant rats in late gestation in comparison to the effect in non-pregnant rats (Castro et al., 1993). It is, therefore, still unclear whether or not changes in the cardiovascular reflex system are related to the potentiation of the hypotensive response of adrenomedullin in pregnant rats.

Another possibility for the potentiation of the hypotensive response of adrenomedullin is that of changes in adrenomedullin receptor during pregnancy. However, there has only been one report describing expression of the adrenomedullin receptor during pregnancy up to now (Upton et al., 1997). In this report, the density of the adrenomedullin binding site was demonstrated to be higher in the pregnant (20 days of pregnancy) uterus than in the non-pregnant uterus, indicating the upregulation of this receptor in late gestation. However, there is no precise information concerning the expression of the adrenomedullin receptor in pregnant rats at various gestational stages. In the present study, we, for the first time, demonstrated an increased adrenomedullin receptor mRNA level in the aorta during late gestation, but not in early and mid-gestation. In addition, we demonstrated that the increased mRNA stability may contribute to an accumulation of adrenomedullin receptor transcript during pregnancy. It is, therefore, possible that the potentiation of the depressor response to adrenomedullin during late gestation is related to the increased adrenomedullin receptor expression. However, we did not perform a receptor binding study. Therefore, further studies are desirable to clarify the precise mechanisms for the actions of adrenomedullin in pregnant rats.

It is well-known that the maternal plasma cortisol concentrations increase during pregnancy in several species (Demey-Ponsart et al., 1982; Keller-Wood, 1996). In addition, there is a recent report that glucocorticoid upregulates the expression and secretion of adrenomedullin in vivo (Hattori et al., 1998). The possibility of regulation of adrenomedullin receptor gene expression by glucocorticoid can, therefore, not be ruled out. However, there is no report concerning the regulation of adrenomedullin receptor gene expression by glucocorticoid.

Adrenomedullin is thought to dilate blood vessels by increasing the cAMP level in the smooth muscle cells of the vascular wall. However, Itahara et al. (1994) found the hypotensive activity of adrenomedullin in rats to be diminished by NO synthase inhibitor, a finding that suggests endothelium-dependent vasodilation by adrenomedullin. Therefore, the mechanism of the hypotensive effect of adrenomedullin may be related, in part, to the NO system. In addition, it has recently been suggested that an increase in NO synthase occurs during late gestation (Nathan et al., 1995). It is, therefore, possible that adrenomedullin may act synergystically with NO. Namely, adrenomedullin accelerates NO production in late gestation. However, further investigation is needed to clarify this point.

5. Conclusion

The present results suggested that the change in the depressor response to adrenomedullin, which occurs at term in pregnant rats, may be mediated by changes in adrenomedullin receptor gene expression rather than by sex hormones.

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