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EXPRESSION OF BIOLOGICALLY ACTIVE RECEPTORS FOR NATRIURETIC PEPTIDES IN THE HUMAN UTERUS DURING PREGNANCY

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SUMMARY: Human amnion cells secrete a large amount of brain natriuretic peptide (BNP). In the present study, to elucidate the possible roles of amniotic BNP during the course of human pregnancy, the tissue distributions of biologically active receptors of natriuretic peptides, atrial natriuretic peptide (ANP)-A receptor and ANP-B receptor in the human uterus during pregnancy were investigated by Northern blot analysis and an *in vitro* guanosine 3',5'-cyclic phosphate (cGMP) generation assay, the second messenger of natriuretic peptides, using crude membrane preparations. Both ANP-A and ANP-B receptor mRNAs were detected in the tissues of decidua vera, chorion laeve, myometrium and placenta. The *in vitro* cGMP generation assay revealed that the expression of ANP-A receptor was more prominent than that of ANP-B receptor in these tissues. On the other hand, in amniotic tissue only ANP-B receptor mRNA was weakly expressed but ANP-A receptor mRNA was not detected. © 1994 Academic Press, Inc.

The natriuretic peptide family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (1). BNP and ANP are secreted into the circulation from the heart as cardiac hormones to regulate blood pressure and body fluid homeostasis in both nonpregnant and pregnant patients with hypertension (2 - 4) and in newborns with fetal distress syndrome (5).

Recently, we found that a high concentration of BNP (approximately 100 pM), 50-fold higher than that in maternal or fetal plasma, was present in human amniotic fluid in the first and second trimester of pregnancy and that the BNP level in amniotic fluid markedly decreased in the third trimester of pregnancy (6). We obtained evidence suggesting that BNP synthesis in amnion cells is regulated by cortisol, epidermal growth factor and transforming growth factor-β, substances present in human amniotic fluid at term, and that BNP secreted from amnion cells reaches intrauterine tissues adjacent to amniotic cavity (6,7). Potvin et al. reported that

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Abbreviations used: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; cGMP, 3',5'-cyclic phosphate.

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ANP and guanosine 3',5'-cyclic phosphate (cGMP), the second messenger of natriuretic peptides, exerted a tocolytic effect on the uterus during pregnancy. These findings lead us to a hypothesis that BNP, secreted from human amnion cells, acts as a relaxant of the uterus during pregnancy.

Natriuretic peptides exert their biological activities through specific receptors. The natriuretic peptide receptors are composed of two biologically active receptors, ANP-A receptor and ANP-B receptor, and one clearance receptor. Both ANP-A and ANP-B receptors have a guanylate cyclase domain in their intracellular structure, which generate cGMP as a second messenger. The ligand selectivity of the ANP-A receptor is ANP≥BNP>>CNP, and that of the ANP-B receptor is CNP>ANP≥BNP (9). Binding sites for ANP were reported to be present in human placental tissue (10). However, the distribution of natriuretic peptide receptors in other tissues in the human uterus during pregnancy has not been fully elucidated.

On the other hand, CNP is secreted from vascular endothelial cells (11), and has been reported to be present in the endometrium of nonpregnant porcine uterus (12).

In the present study, to examine our hypothesis, we investigated the expressions of ANP-A and ANP-B receptors in uterine tissues in the second trimester of pregnancy and at term using Northern blot analysis and an *in vitro* cGMP generation assay with crude membrane preparations.

MATERIALS AND METHODS

Reagents: Human ANP, human BNP and CNP were purchased from Peptide Institute, Inc. (Minoh, Japan). All other reagents used were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Tissue preparation: Placental tissues and fetal membranes at term were obtained at normal vaginal deliveries. Myometrium and fetal membranes in the second trimester were obtained at the time of therapeutic abortions or hysterectomy for gynecological diseases. The amnion was manually separated from the chorion laeve. Decidual tissue was bluntly separated from the chorion laeve. The tissues thus separated were immediately frozen in liquid nitrogen and stored at -70 °C until assay. In each case, patient's consent was obtained after full explanation of the nature of the present study.

Northern blot analysis: Northern blot analysis was carried out as previously described (13). Briefly, the human ANP-A receptor cDNA probe (323 bp) was prepared by cDNA synthesis and PCR using total RNA isolated from the human thoracic aorta and the following oligonucleotide primers; sense, 5'-GAGAACATCACTCAGCGGATG-3', and antisense, 5'-CCGAGCAAGGAGAGGCTGCCC-3', based on the published human ANP-A receptor sequence (14). The human ANP-B receptor cDNA probe (500 bp) was prepared by the same procedures using the following oligonucleotide primers; sense, 5'-GGGAGAGTCTCCGT GCAGGC-3', and antisense, 5'-AATAGGCCGTCCCGTCCACC-3', based on the reported human ANP-A receptor sequence (15). The total RNA extracted from these tissues by guanidinium thiocyanate cesium chloride method was electrophoresed on a formamide / 1.2 % agarose gel and transferred on to a nylon membrane filter. The filter, hybridized with the ^{32}P-labeled probes, was washed and autoradiographed.

Guanylate cyclase assay with crude membrane preparations: Frozen tissues were thawed and homogenized with a Polytron in 5 volumes of ice-cold 20 mM potassium phosphate buffer, pH 7.4, with 5 μg / ml EDTA, 10 μg / ml aprotinin (Ohkura Pharmaceutical Co., Kyoto, Japan), 10 μg / ml leupeptin (Peptide Institute), 10 μg / ml pepstatin-A (Peptide Institute), and 100 μg / ml phenylmethylsulfonyl- fluoride (Boehringer Mannheim, Mannheim, Germany). All procedures were conducted at 4 °C. The homogenates were centrifuged at 600 x *g* for 5 min, and the supernatants were then centrifuged at 37,000 x *g* for 30 min. The resultant particulate fractions were washed three times in the same buffer. The crude membrane fractions

thus prepared (2-10 μg protein / tube) were applied to 100 μl of guanylate cyclase assay reaction mixture, consisting of 50 mM Tris-HCl buffer (pH 7.6) containing 0.5 mM isobutylmethylxanthine, 0.1 % bovine serum albumin, 4 mM $MnCl_2$, 1 mM guanosine 5'-triphosphate (GTP), 15 mM creatine phosphate, and 20 μg of creatine phosphokinase (163 units / mg) with or without natriuretic peptides (10^{-8} M - 10^{-6} M) (16). These assay mixtures were incubated for 10 min at 37 °C, after which the reactions were terminated by adding 100 μl of ice-cold 12 % trichloroacetic acid (TCA). After extraction of TCA by diethylether, the reaction mixtures were lyophilized and the amounts of cGMP generated were measured by RIA as previously described (17).

RESULTS

Figure 1 shows Northern blot analysis of total RNA from amniotic tissues in the second trimester and at term, chorion laeve at term, decidua vera tissues in the second trimester and at term, placental tissue at term and myometrium in the second trimester. In the tissues of chorion laeve, decidua vera, placenta and myometrium, mRNA bands hybridizing to both the human ANP-A receptor and ANP-B receptor cDNA probes were detected with expected sizes of approximately 4.0 kilobases as previously described (9,13).

On the other hand, no band corresponding to ANP-A receptor mRNA was observed in amniotic tissues in the second trimester or at term, although a weak bands hybridizing with the ANP-B receptor probe were detected in these tissues.

Generation of cGMP, the second messenger of natriuretic peptides, was examined using crude membrane preparations of these tissues by the addition of ANP, BNP or CNP (10^{-8} M - 10^{-6} M). The guanylate cyclase activity was not augmented by addition of ANP, BNP or CNP to the crude particulate preparations of amniotic tissues (Figure 2, A and B). In the membrane preparations of the decidua vera, placenta and myometrium, however, cGMP generation was markedly augmented by addition of ANP and of BNP, corresponding to activation of the ANP-A receptor particulate guanylate cyclase (9). On the other hand, cGMP generation was only weakly augmented by CNP. These results together indicate that ANP-A receptor, which has

Fig.1. Tissue distribution of ANP-A and ANP-B receptor mRNAs in the human uterus during pregnancy.
Northern blot analyses of human ANP-A receptor mRNA and ANP-B receptor mRNA from amniotic tissue in the second trimester (lanes 1,2)and at term (lanes 3,4),chorion laeve at term (lane 5), decidual tissue in the second trimester (lane 6) and at term (lane 7), myometrium in the second trimester (lane 8), and placenta at term (lane 9) were performed as described in MATERIALS AND METHODS. 20 μg of total RNA was loaded in each lane. 28S rRNAs in agarose gel were visualized by ethidium bromide staining. kb; Kilobase.

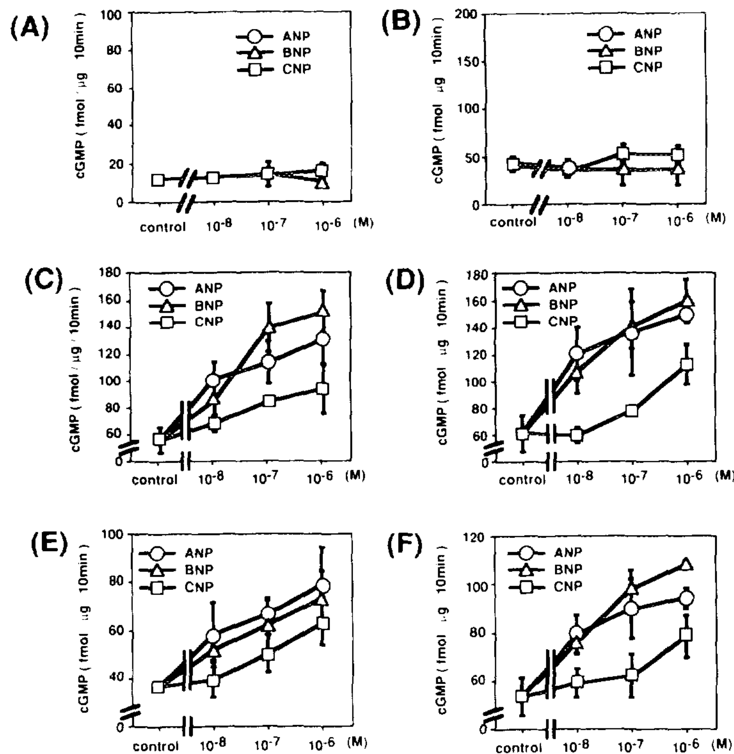

Fig.2. Effects of ANP, BNP and CNP on cGMP production in crude membrane preparations.

Crude membrane preparations of amniotic tissue in the second trimester (A) and at term (B), decidual tissue in the second trimester (C) and at term (D), myometrium in the second trimester (E), and placenta at term (F) were incubated for 10 min at 37 °C in the reaction mixture in the presence or absence of ANP, BNP and CNP (10^{-8} - 10^{-6} M) as described in MATERIALS AND METHODS. In each figure, open circles, open triangles and open squares indicate supplementation with ANP, BNP and CNP, respectively. Values are means ± SEM of quadruplicate assays. Experiments were repeated at least three times to confirm the results, and the representative results are shown in the figure.

high affinity to ANP and BNP but not to CNP, is mainly activated, and that the ANP-B receptor, which has high affinity to CNP but not to ANP or BNP, is only weakly activated (Figure 2, C, D, E and F). Thus, the ANP-A receptor appeared to be the major component of particulate guanylate cyclases in these tissues. These findings were compatible with the distribution patterns of ANP-A receptor and ANP-B receptor mRNAs in the second trimester and at term.

DISCUSSION

Human amnion cells secrete a large amount of BNP (6). In the present study, ANP-A receptor, the receptor specific for ANP and BNP, was not detected in amniotic tissues in the second trimester and at term, indicating that BNP secreted from amnion cells might not act on

amnion cells themselves. We previously observed a high concentration of BNP in the extra-embryonic coelom, indicating that BNP is also secreted from amnion cells in the direction of the maternal side (6). On the other hand, in the present study, the expression of ANP-A receptor was identified in tissues of the chorion laeve, decidua vera, and myometrium by both in vitro cGMP generation assay and Northern blot analysis. These findings support our hypothesis that BNP secreted from amnion cells acts in a paracrine manner on tissues adjacent to the amniotic cavity in the human uterus during pregnancy, generates cGMP, and finally plays some role(s) in the maintenance of pregnancy.

Word et al. demonstrated that 8-bromo-cGMP, a cell membrane-permeable derivative of cGMP, decreased the magnitude and frequency of spontaneous contraction of human myometrium (18). Moreover, in our preliminary study, rat BNP as well as 8-bromo-cGMP inhibited the prostaglandin $F_{2\alpha}$-induced contraction of rat pregnant myometrium (Itoh H and Sagawa N, unpublished observations). Since cGMP is the second messenger of BNP, these findings strongly suggest that BNP, secreted from amnion cells, exerts tocolytic effects on pregnant myometrium, especially in the first and second trimester of pregnancy when BNP secretion from amnion cells is high, and that such an inhibitory effect on myometrial contraction is attenuated at term when BNP secretion from amnion cells markedly decreases (6,7).

In the present study, the ANP-A receptor was detected in human decidua vera tissue, and BNP augmented the cGMP generation in this tissue. Although the roles of cGMP and BNP in human decidua vera tissue have not yet been clarified, the effects of cGMP and BNP on the function of decidual cells are currently under investigation in our laboratory.

The results of the present study also demonstrated the expression of ANP-B receptor, which has high affinity for CNP, in the human uterus during pregnancy. Chrisman et al. reported that CNP-like immunoreactivity (CNP-LI) was present in the endometrium of nonpregnant porcine uterus, and that ANP-B receptor was expressed in nonpregnant porcine and rat uterus (12). In our preliminary study, CNP-LI was below the detection limit in human amniotic fluid (less than 0.3 fmol/ml), and in human intrauterine tissues such as the placenta, amnion, chorion, decidua, pregnant myometrium and nonpregnant endometrium (less than 30 fmol/g wet weight) (Itoh H and Sagawa N, unpublished observations). However, we previously found that CNP secretion from vascular endothelial cells was markedly stimulated by the addition of TGF-β, tumor necrosis factor-α and lipopolysaccharide (19). Thus, there remains a possibility that under specific conditions such as with intrauterine infection, the CNP secretion from intrauterine tissues including uterine vascular endothelial cells, is stimulated by these substances. In such cases, the increased level of CNP may act on ANP-B receptors in decidua and myometrium and play some role(s) in the maintenance of pregnancy.

Recently, clinical applications of natriuretic peptides have been proposed. For example, the administration of BNP has been reported to improve congestive heart failure (20). The results of the present study suggest that natriuretic peptides may be useful as tocolytic agents to prevent preterm uterine contraction. Another possibility is an application of a specific antagonist for natriuretic peptide receptors such as HS-142-1 (21). For example, the administration of

HS-142-1 may block cGMP generation by BNP and increase the sensitivity of uterine myometrium to uterine contractics such as prostaglandin or oxytocin, and consequently induces uterine contraction. However, further investigations including *in vivo* animal studies with these substances are required before these possible clinical applications can be confirmed.

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