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The Effect of a Potent Oxytocin Antagonist, Antag I, on *In Vivo* Uterine Contractions in Response to Exogenous Oxytocin and on Uterine Oxytocin Receptor Number and Affinity

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옥시토신 길항제, Antag I이 옥시토신 투여에 따른 자궁수축과 자궁의 옥시토신 수용체 수 및 친화력에 미치는 영향

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요 약

우수성이 이미 입증된 oxytocin antagonist-I(AI)이 발정된 쥐의 자궁내 옥시토신 수용체 수와 결합친화력에 어떤 영향을 미치는 지를 알아보는 것이 본 연구의 목적이였다. 마취된 흰쥐들에게 5 μg의 AI를 투여하였으며, 30분과 4시간후에 도살하였다. 자궁조직을 회수하여 잘게 잘라 냉동시킨 다음 다시 자궁조직을 분쇄한 후 단계적인 초고속 원심분리를 거쳐 옥시토신 수용체가 있는 세포막을 추출하였다. 옥시토신 수용체 분석은 높은 활동성을 보이는 옥시토신 길항제와 방사선 동위원소가 붙지 않은 옥시토신이 경쟁하는 포화상태에서 실시하였다. 옥시토신 수용체의 수와 결합친화력은 nonlinear curve fitting 방법에 의해 계산되었다. 연구 결과, AI이 투여된 흰쥐들은 수용체의 수와 결합력에 있어서 control과 유의한 차이를 보이지 않았다($p>0.05$).

결론적으로 AI은 옥시토신 수용체의 수와 결합친화력을 변화시키지 않고 다만 옥시토신과 경쟁력으로 억제하는 작용을 옥시토신 수용체에서 나타낸다는 것을 이 연구에서 입증하였다.

I. INTRODUCTION

The role of oxytocin(OT) during parturition in primates has not been clearly defined due to inconsistent reports on the changes in plasma oxytocin levels. However, some studies support an involvement of oxytocin during the labor process as demonstrated by an increase in the myometrial oxytocin receptor number(Fuchs et al., 1983), by the increase in sensitivity of uterine myometrium to oxytocin(Fuchs and Poblete, 1970) and by the increase in uterine oxytocin gene expression(Lefebvre et al., 1992). Furthermore, preterm labor is associated with a significant increase in myometrial sensitivity to oxytocin while post-term labor is associated with decreased sensitivity (Takahashi et al., 1980).

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If oxytocin is important in preterm labor, an oxytocin antagonist (OTA) would be of great clinical significance.

We have previously reported that oxytocin antagonist-I(AI) can inhibit in vitro and in vivo oxytocin induced uterine contractions in the rat and baboon(Wilson et al., 1990a; Wilson et al., 1990b). It is assumed that AI is blocking uterine contractions via competitive inhibition at the oxytocin receptor. However, it is possible that AI is having other effects on the receptor. Therefore, the purpose of this study was to determine if AI in vivo alters uterine oxytocin receptor number and/or binding affinity in the estrous rat.

Ⅱ. MATERIALS AND METHODS

1. Animals

Holtzman rats(Holtzman Co., Madison, Wisconsin)were used in this study. Animals were housed in rooms with controlled light cycles (14 hours light/10 hours dark) and fed as desired. This study was approved by the University of Illinois Animal Care Committee.

2. Oxytocin antagonist

The synthesis and potency estimates have been previously described by our laboratory(Wilson et al., 1990a; Flouret et al., 1991). AI has the following structure: [Beta-mercapto-beta, beta-cyclopentamethylene propionic acid[1], D-Trp[2], Phe[3], Ile[4], Arg[8]]-oxytocin.

3. Animal cannulation

Rats in natural estrus were anesthetized with chloral hydrate(500 mg/kg) intraperitoneally. A catheter(PE-50) was placed into the jugular vein for infusing AI(5 μg)or control (saline) as a bolus injection. Uterine contractions were monitored as previously described(Wilson et al., 1990a). Briefly, following a mid-ventral abdominal incision a PE-50 balloon-tipped, water filled cannula was placed into one uterine horn at the ovarian end. Integrated intrauterine pressure changes were measured over 10 minutes using Grass Polygraph. Animals were sacrificed by overdosing with chloral hydrate.

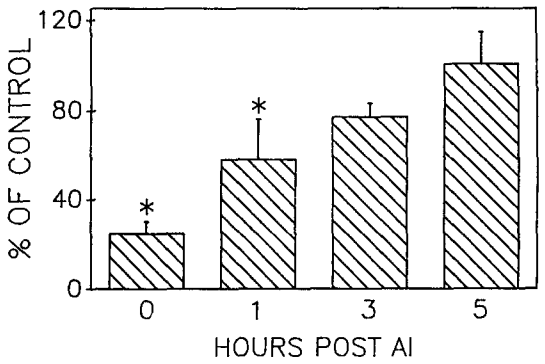

Fig. 1. Depicted is the inhibitory effect of oxytocin antagonist-I(AI) on *in vivo* uterine contractile response in estrous rats expressed as percentage of control. The animals were given a bolus infusion of 5 μg of AI or control and challenged with a bolus infusion of 100 mU of oxytocin at 0, 1, 3 and 5 hours. Uterine contractile activity was integrated for 10 minutes following the oxytocin infusions. *Significantly different between AI treated animals and controls($p<0.05$). Mean± SEM. N=6 at each time point.

4. *In vivo* oxytocic bioassay

Animals were given a bolus iv injection of 5 ug of AI or control and at 5 minutes(0 hour), 1, 3 and 5 hours later an iv bolus injection of 100mUs of oxytocin was given and uterine activity monitored for 10 minutes. Six rats were used for each treatment for a total of 12 rats.

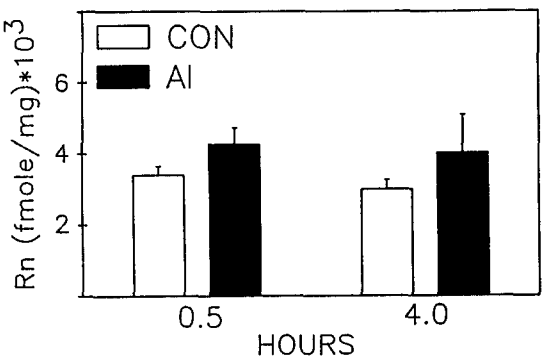

Fig. 2. The oxytocin receptor number after oxytocin antagonist-I(AI) infusion in estrous rat was not different from controls($p>0.05$) at either 0.5 or 4 hours time point. Rn=oxytocin receptor number. Mean±SEM. N=6 for each treatment group.

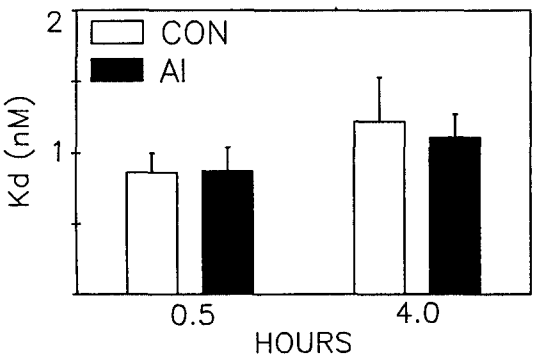

Fig. 3. The effect of oxytocin antagonist-I(AI) infusion on oxytocin receptor dissociation constant in estrous rat was not significantly different from control ($p>0.05$) at either time point studied. Kd=dissociation constant. Mean±SEM. N=6 at each treatment group.

5. Oxytocin receptor assay

Uterine tissue was obtained at 0.5 and 4 hours following 5 μg AI or control iv bolus administration. These time point were chosen based on the *in vivo* oxytocin challenge test results shown in Fig. 1 which are times during and after AI was still effective. At 0.5 hrs AI significantly inhibited the uterine response to exogenous OT but by 4 hours the effect disappeared. There are 6 rats at each time point and treatment for a total of 24 rats used in this study.

The methodology of the assay has been described previously(Pak et al., 1994). In brief, the uterine tissue from sacrificed estrous rat was cut into small pieces with scissors, frozen on dry ice, and stored at −70°C until analysis. The uterine tissue was homogenized in 5 mM Tris buffer containing PMSF(protease inhibitor) over ice. The homogenate was filtered through a gauze and the filtrate was centrifuged at 1000 × g for 1.5 minutes at 4°C. The supernatant was centrifuged at 40,000 × g for 30 minutes and the cell membrane containing pellet resuspended in 10% sucrose. To perform density gradient centrifigation, the 10% sucrose membranes were placed on top of 35% sucrose and centrifuged for 30 minutes in a swing bucket rotor at 105,000 × g.

The membranes at the interface of the 10%/35% sucrose were removed and resuspended in Tris buffer containing EDTA for 30 minutes. This procedure removes divalent cations and results in dissociation of endogenously bound oxytocin to the receptor. The mixture was centrifuged for 15 minutes at 100,000 × g and the pellet was resuspended in Tris, PMSF, Mg buffer by sonication

The binding assay consists of 0.05ml(10,000cpm) of $d(CH^2)_5[Tyr(Me)^2, Thr^4, Orn^8[^{125}I]Tyr^9\text{-}NH_2]$-Vasotocin(DuPont Company, Wilmington, DE; 2200 Ci/mmol), 0.05ml of oxytocin(Calbiochem Behring Corp., LaJolla, CA), membrane oxytocin receptor and 50 mM Tris buffer containing 0.5% trypsin inhibitor, 100mM

PMSF, 1% BSA and 20 mM MgCI. The incubation in ultraclear minitubes (5 × 41 mm) was performed for 30 minutes at 30°C. This was followed by centrifugation for 30 minutes at 105, 000 × g. The resulting membrane pellet was counted for cpm in gamma counter.

The receptor number and dissociation constants(kd) were determined by nonlinear curve-fitting techinques using McPherson's (1985) EBDA and Munson and Rodbard's (1980) LIGAND programs for saturation and competition analysis.

Ⅲ. DATA ANALYSIS

Data was analysized by two way analysis of variance with interation(Norusis, 1988).

Ⅳ. RESULTS

Fig. 1 depicts the result of the inhibitory effect of AI on oxytocin induced uterine contractions *in vivo* as percentage of control. AI showed its inhibitory effect up to 2 hours but that effect disappeared by 4 hours. Based on this result, we designed the present study to look at the oxytocin receptor number and dissociation constant at the 0.5 and 4 hours. Differences in the Main effects (Treatment and Time) or an interaction were not significant ($p>0.05$). The results of Treatment (ie AI vs Control)on oxytocin receptor number are shown in Fig. 2. while that on binding affinity are presented in Fig. 3.

Ⅴ. DISCUSSION

The present study shows no significant effect of AI on oxytocin receptor number and/or oxytocin receptor binding affinity when compared with controls. Therefore, these results suggest that AI is acting via competitive inhibition of the oxytocin receptor. It is possible that AI is also having a post-receptor effect. However, the *in vitro* oxytocin bioassay studies do not support this possibility since the rat uterus returns to normal responsitivity after washing out the AI from the organ bath.

The development of a potent oxytocin antagonist will be useful for studying the contribution of endogenous oxytocin to nocturnal and labor uterine contractions during pregnancy. The maximum oxytocin receptor number occurs at the end of pregnancy in the rat (Fuchs et al., 1983). correlating with the increase in uterine sensitivity to oxytocin at the time of labor. Although Lefebvre et al.(1992) demonstrated that oxytocin mRNA in the uterus and placenta increased as delivery approaches, the expression of OT appears to be minimal thus questioning the contribution to uterine activity at delivery. AI is a potent inhibitor of oxytocin induced uterine contractions as evidenced by inhibition of uterine contractions induced by exogenous oxytocin *in vitro* and *in vivo,* by inhibition milk let-down and by disruption labor. Furthermore, AI inhibited *in vitro* uterine contractions induced by oxytocin in myometrial tissue obtained by C-section at term pregnancy in humans (Wilson et al., 1990a). Thus, AI appears to be a potent OTA which our laboratory has shown is about 2. 4 fold more potent than Atosiban by oxytocic bioassay the latter which is currently undergoging clinical trials throughout the world.

Ⅵ. ABSTRACT

The purpose of the present study was to determine the *in vivo* effect of oxytocin antagonist-I(AI) on uterine oxytocin receptor number (Rn) and/or binding affinity (Kd) in the estrous rat. Anesthetized rats were given a bolus infusion of control or 5μg of AI and sacri-

ficed 0.5 and 4 hours later. The uterine tissue was removed, trimmed and frozen. Membrane oxytocin receptors were isolated after homogenization of uterine tissue and differential ultracentrifugation. The oxytocin receptor assay was performed by saturation with cold oxytocin competion with a high specific activity oxytocin antagonist. Rn and Kds were determined by nonlinear curve fitting methods. No differences($p>0.05$) between the AI and control treated animals in either oxytocin receptor number or binding affinity was detected in this study. These data suggest that the major mode of action of AI is via competitive inhibition at the uterine oxytocin receptor and not by altering receptor number or binding affinity.

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