Study of Oxytocin Receptor in Human Myometrium Using Highly Specific ^3H -Labeled Oxytocin

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Synopsis

A highly specific tritium labeled oxytocin (^3H -OT) was synthesized utilizing the method of catalytic substitution of halogen for hydrogen. The specific activity of ^3H -OT was 19 Ci/mM and the biologic activity was 350 U/mg, which was sufficient for the OT radioreceptor assay. The maximum % uptake of ^3H -OT in the human myometrium was observed in $20,000\times g$ pellets under the optimal conditions of pH 7.4, at 20°C and the incubation time of 90 min and it was augmented in the presence of Mn^{++} . It was also observed from the Scatchard plot, that the binding site of OT in the human myometrial specimens was a single type within the range of OT concentration of 0.4 nM to 1.6 nM. The dissociation constants (K_d) and the number of binding sites (NBS) showed a relative increase as gestation advance. The apparent K_d of term pregnancies was 1.25×10^{-9} M regardless of the presence or absence of labor pains, while the NBS of term pregnancies with and without labor pain was 1.2×10^{-12} and 4.7×10^{-12} moles/mg protein, respectively.

It is well known that the uterine myometrial contractions are induced by the administration of oxytocin (OT) which is thought to play the main role in controlling the onset of labor, namely parturition. We know of no reports concerning the mechanism of the action of OT in the uterus during parturition, even though there have been some studies on plasma OT levels (Chard *et al.*, 1971; Kumaresan *et al.*, 1974; Dawood *et al.*, 1977) and on the specific binding site for ^3H -OT in the uterus (Soloff *et al.*, 1974). One of the reasons for such a small number of reports is the lack of isotopically labeled OT with a high specific activity as well as high biologic activity. Therefore, we attempted to synthesize a ^3H -labeled OT by the method of catalytic substitution of di-iodo OT for tri-

ated OT according to the modified methods of Agishi and Dingman (1965) and Morgat *et al.* (1970). Using this newly synthesized ^3H -OT with a specific activity of 19 Ci/mM and with biologic activity of 350 U/mg, several investigations were made on human myometrial specimens including term pregnancies with and without labor pain in an attempt to clarify the pregnancy related dynamics of OT receptors.

Materials and Methods

Reagents and Apparatus

Standard OT (Sanabo, Austria) was stored in 1 mM sodium acetate buffer, pH 4.6. Ion exchange resin AGII-A8 (50-100 mesh) and Biogel P₂ (400 mesh, Bio-Rad Lab.) were used for the column chromatography (CC). Cellulose thin layer chromatography plates (Art 5728, TLC) were obtained from E. Merck A. G., Darmstadt. 5% Pd/CaCO₃ was from Japan Engelhard and all reagents from Wako Pharmaceuti-

cal Co. were of analytical grade. 1 mM of phosphate buffer, pH 6.4, Tyrode's solution, pH 7.6 and 50 mM Tris-maleate buffer, pH 7.4 were prepared. Scintillation fluid was made by dissolving 275 g of POPOP, 6 g of DPO and 100 g of naphthalene in 1,000 ml of dioxane. All glass or teflon potter homogenizer, liquid scintillation spectrometer (Mark III 6880, Searle Analytical Inc.), autowell gamma counter (Aloka JDC 771), thin layer chromatogram scanner (Aloka TLC-2B) and ultraviolet spectrometer (Type 323, Hitachi) were used.

Purification of Standard Oxytocin

Purification of the standard OT was developed on cellulose TLC, using a solvent system of n-butanol: acetic acid: water (75:10:25) and the fractions of which Rf values were from 0.57 to 0.71 were collected and extracted with 1 mM phosphate buffer. The extract was then passed through a glass filter (G4) and a millipore (1 μ mesh, Amicon Corp.). The purified OT showed 470 U/mg of biologic activity and was subjected to the following experiment.

Preparation of ^3H -labeled Oxytocin

^3H -labeled OT was synthesized by the modified method of Glover *et al.* (1967) and Morgat *et al.* (1970). Standard OT was dissolved in 1 mM of phosphate buffer and 0.1 M ICl in methanol was titrated in over a period of 10 min at 4°C. After adding 0.1 M sodium thiosulfate, the mixture was transferred to the ion exchange resin CC (8 $\times$ 50 mm) to remove the inorganic salts. The di-iodinated OT fraction was further submitted to Biogel P₂ CC (20 $\times$ 400 mm) using 1 mM phosphate buffer as an effluent with the flow rate of 0.2 ml/min. The protein concentration of the fraction, 2.8 ml each, was determined by the Folin method (Lowry *et al.*, 1951) as well as UV spectrum. 1 g of ion exchange resin was added to the obtained fraction and the mixture was stirred vigorously. The supernatant of the mixture obtained by centrifugation at 3,000 rpm was condensed *in vacuo* and frozen with liquid nitrogen in a ^3H reaction vessel (Hata *et al.*, 1977) after 5% Pd/CaCO₃ was added as catalyst. The excess ^3H gas was perfused *in vacuo* and the reactant was stirred for 40 min at 24°C with a gas pressure of 250 mmHg. The reactant was passed through a millipore and the radiochemical purity was tested until the radioactivity of the product became constant. The crude ^3H -OT was further developed on Biogel P₂ CC with 1 mM acetate buffer and the protein concentration as well as the radioactivity of each 2.8 ml fraction was determined.

Preparation of the Uterine Particulate Fraction

Uterine tissues were obtained from normally menstruating women who had undergone hysterectomy because of myomas or other benign gynecologic problems. First trimester uterine tissues were ob-

tained from the surgical cases requiring hysterectomy which were complicated by pregnancy. Term myometria were obtained during the elective or definitive cesarean section from the anterior portion of the lower segment. The specimens were immediately placed in an ice-chest and the subsequent procedures were all carried out at 4°C. After removing the endometrium, serosal layer and myomatous tissue, the myometrial specimens were minced and homogenized in 5 vol of Tyrode's solution in a potter homogenizer. The homogenate was initially centrifuged at 1,000 $\times g$ for 15 min, then the supernatant was further centrifuged at 20,000 $\times g$ for 15 min, followed by centrifugation at 105,000 $\times g$ for 15 min. All particulate fractions and the final 105,000 $\times g$ supernatant were used in the radioreceptor assay. The 20,000 $\times g$ pellets were treated according to the method reported by Soloff *et al.* (1974). The specimens were centrifuged at 20,000 $\times g$ for 15 min and the pellets were washed with 2 vol of Tris-maleate buffer containing 5 mM of MnCl₂ and 0.1% of gelatin. After recentrifugation at 20,000 $\times g$ for 15 min, the pellets were suspended in 2 vol of Tris-maleate buffer to give a protein concentration ranging between 1.5 mg/ml to 4.0 mg/ml. The incubation was performed as follows; 100 μl of the specimen, 7,000 dpm of ^3H -OT and sufficient standard OT to make a total volume of 500 μl of Tris-maleate buffer were incubated at 20°C for 90 min and the reaction was terminated by centrifugation at 20,000 $\times g$ for 15 min at 4°C. The radioactivity of the pellets dissolved in 200 μl of 1 N NaOH as well as that of the supernatant were both determined simultaneously. Scatchard analysis (Scatchard, 1949) was employed for the determination of apparent dissociation constants (Kd) and the number of binding sites (NBS).

Results

Commercial grade OT developed on cellulose TLC showed three spots with Rf values of 0.39, 0.48 and 0.64 the last of which showed a UV spectrum of 275 nm (λ max) at pH 4.6 and a biologic activity of 407 U/mg by the avian depressor method. After the Biogel P₂ CC, the protein concentration of each 2.8 ml fraction of di-iodinated OT was determined. The shift of spectrum was observed in fractions 31 to 35, which showed maximal protein concentrations. The shift was from 275 nm (λ max) at pH 4.6 to 312 nm (λ max) at pH 7.0 (Fig. 1). In another experiment, it was recognized

that the specific activity of ^{125}I -labeled OT was 3.6 Ci/M which was twice as high as that of ^{125}I Cl, 1.8 Ci/M. And the R_f value of di-iodinated OT developed on TLC was 0.82 while that of the standard OT was 0.68. The R_f value of the crude ^3H -OT

developed on TLC was almost equal to that of the standard. Adjacent spots, however, were found on TLC that could not be eliminated by the repeated TLC procedure, therefore, further purification was carried out on Biogel P_2 CC, the fractions of whose

Fig. 1. Ultraviolet spectra of oxytocin and di-iodo oxytocin. The shift of spectra was observed from 275 nm (λ_{max}) at pH 4.6 to 312 nm (λ_{max}) at pH 7.0.

Fig. 2. Elution pattern of ^3H -labeled oxytocin from a Biogel P_2 column chromatography (20×400 mm) using 10^{-3} M acetate buffer, pH 4.6 as elution buffer. The protein concentration and radioactivity of each 2.8 ml fraction were determined.

^3H activity corresponded to the optical density were collected (Fig. 2). These fractions were further developed on TLC and Biogel P₂ CC (Fig. 3). It was proved that ^3H -OT finally obtained was 3.8% and had 19 Ci/mM of specific activity and 350 U/mg of biologic activity. This synthesized ^3H -OT was kept at -70°C dissolved in 1 mM acetate buffer.

In order to determine the optimal conditions for the OT radioreceptor assay, the

% uptake of ^3H -OT was firstly estimated in $1,000\times g$, $20,000\times g$ and $105,000\times g$ pellets as well as in $1,000\times g$, $20,000\times g$ and $105,000\times g$ supernatant of the non-pregnant myometrial specimens under varying pH, temperature and the incubation time. The maximum uptake of 7,000 dpm ^3H -OT was observed in the $20,000\times g$ pellets at about 80% bound/mg protein under the incubation conditions of pH 7.4, at 20°C for 90 min in the volumes of Tris-maleate

Fig. 3. Identification of ^3H -oxytocin from cellulose thin layer chromatography using solvent system, $n\text{-BuOH} : \text{CH}_3\text{COOH} : \text{H}_2\text{O}$ (75 : 10 : 25).

Fig. 5. Effects of various divalent cations on ^3H -oxytocin binding to the $20,000\times g$ pellets of human nonpregnant myometrium. Tris-maleate buffer with 5 mM of Ca^{++} , Cu^{++} , Mg^{++} and Mn^{++} were prepared and incubation was performed.

Fig. 4. Effects of incubation time, temperature and pH on ^3H -oxytocin binding to the $20,000\times g$ pellets of human nonpregnant myometrium. $100\ \mu\text{l}$ of specimen was incubated with 7,000 dpm of ^3H -oxytocin to a total volume of $500\ \mu\text{l}$ of Tris-maleate buffer with various incubation times, temperature and pH.

Fig. 6. Competitive radioreceptor assay was performed in the $20,000\times g$ pellets of nonpregnant, first trimester, term pregnancies with labor pain and without labor pain. The apparent K_d s and the NBS were estimated from Scatchard analysis.

Fig. 7. Comparison of the NBS during gestation and at term by X-intercept of the Scatchard plot.

buffer as shown in the Fig. 4. The % uptake increased about 40% in the presence of Mn^{++} and 10% with Mg^{++} , while no potentiation was observed in the presence of Ca^{++} or Cu^{++} (Fig. 5). The % uptake to the $20,000\times g$ pellets was determined and compared in nonpregnancy, first trimester pregnancies and term pregnancies with and without labor pains and the values were 12%, 14%, 16% and 30%, respectively. The results of the competitive radioreceptor assay were analyzed by the Scatchard plot, which gave a linear figure with the range of total OT concentrations of 0.4 nM to 1.6 nM. The apparent K_d s of the nonpregnant and the first trimester specimens were $1.66\times 10^{-8}M$ and $1.64\times 10^{-8}M$, and those at term were $1.25\times 10^{-8}M$ regardless of the presence or absence of labor pains (Fig. 6). Also the binding capacities of each specimen expressed as NBS were estimated by the X-Intercept of the Scatchard plot. The NBS of the nonpregnant was 1.0×10^{-12} moles/mg protein, that of the first trimester was 1.1×10^{-12} and those of term with labor pain was 1.6×10^{-12} while that without labor pain was 4.7×10^{-12} (Fig. 7).

Discussion

Radio-iodinated OT has been widely accepted for RIA because of its high specific activity. It is unsuitable, however, for the radioreceptor assay because of the inactivation of biologic activity and the degradation as a result of the iodination of the tyrosyl ring (Thompson *et al.*, 1973). Thus, it would seem reasonable that the 3H -OT rather than radio-iodinated OT is preferable for the OT radioreceptor assay. At present, however, 3H -OT with high specific activity sufficient for the radioreceptor assay is not commercially available, even though Morgat *et al.* (1970) had succeeded in synthesizing a 3H -OT with a specific activity of 36 Ci/mM and biologic activity of 300 U/mg. We attempted to synthesize a 3H -OT according to the method of catalytic substitution of di-iodinated tyrosyl ring for 3H , using the modified method of Agishi and Dingman (1965) and Morgat *et al.* (1970) and succeeded in synthesizing a 3H -OT with a specific activity of 19 Ci/mM and biologic activity of 350 U/mg which is sufficient for the radioreceptor assay. There are several points that should be taken into consideration in this experiment, *i.e.* the purity of

the standard OT and the procedural losses of biologic activity as well as degradation. The relatively low purity and the biologic activity of the standard OT required further purification on cellulose TLC which appeared to decrease the losses in biologic activity of OT, predicted when other methods were employed. And we have obtained an OT of 25% purity with biologic activity of 407 U/mg. It is known that the stability of OT is retained in acidic solutions while iodinated OT tends to lose the I_2 from the tyrosyl ring when the pH is below 5.0. Therefore the reaction step up to the tritiation was carried out in 1 mM phosphate buffer pH 6.4 and thereafter in acetate buffer pH 4.6 to prevent or decrease the degradation of OT. In this study, di-iodination was done applying the method of Glover *et al.* (1967) and ICl used was made by dissolving 2 M KI and KIO_3 in concentrated HCl. Identification of the reactants as di-iodo OT is based on the following observations; The UV spectrum shifted to 312 nm (λ_{max}) at pH 7.0, this agrees with the observation that the peptide containing the iodinated tyrosyl ring shows the specific spectrum shift (Nunez *et al.*, 1962) and the UV spectrum of tyrosine, mono-iodo tyrosine and di-iodo tyrosine are 275, 305 and 312 nm (λ_{max}) at pH 7.0. Secondly the specific activity of ^{125}I -OT was twice as high as that of ^{125}ICl and Rf value of the former on TLC was discriminated from that of the standard OT. Using this di-iodo OT, we carefully performed the catalytic substitution. First, unbound iodine was removed as possible before the catalytic substitution of halogen for hydrogen, because it might inactivate the catalyst. H-I or 3H -I resulting from the residual iodine during the substitution reaction was eliminated by the reaction of $CaCO_3$ to $CaCl_2$, because alkaline solutions in general affect the stability of OT. The actual specific activity of synthesized OT was 19 Ci/mM, while the theoretical was 58 Ci/mM. This

discrepancy may be due to the interchange of some 3H gas with hydrogen in the water; in fact, it is estimated that 50 Ci of 3H gas has been bound to H_2O (about 1.25 Ci/ml), but as the interchange between 3H and halogen is more rapid than that between 3H and hydrogen, the relatively high specific activity of 3H -OT has been obtained.

Using this newly synthesized 3H -OT, several investigations were performed on human myometrial specimens. The % uptake of 3H -OT in the $20,000 \times g$ fraction of the human myometrium was maximal under the incubation condition of pH 7.4, $20^\circ C$ for 90 min, also the uptake was augmented by the presence of Mn^{++} . The 3H -OT binding site, namely the OT receptor activity, was predominantly found in $20,000 \times g$ particulate and was of a single type within the range of OT concentrations of 0.4 nM to 1.6 nM. The binding affinity of OT to OT receptors showed a comparative increase as gestation advanced and so did the NBS value, suggesting that the pregnancy related to the change of the uterine sensitivity to OT may be due to the changes in the receptor or an increase in number of the receptors. In experiments on rats, it was explained that the increased OT receptor affinity together with the increase of NBS during pregnancy might be induced by estrogen (Soloff *et al.*, 1975). In the present study, the NBS value of term pregnancies without labor pain was considerably higher than those in labor, while the Kds did not show any detectable change. This suggests that the OT receptors in the myometrium in the contractile stage may have been saturated with endogenous OT or OT to OT-receptor complex may exert some inhibitory effects on the subsequent binding. The precise mechanism in human uterine tissue, however, still remains to be clarified by the future investigations. We are in the process of setting up a protocol to examine and evaluate these mechanisms.

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