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Studies on Large Mobile Protein appearing in Submandibular Glands of Isoproterenol-treated Rats. V. Further Studies on Some Aspects of Its Formation

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Experiments on *in vivo* (^{3}H)-lysine incorporation into rat submandibular glands after isoproterenol (IPR) treatment were performed. In the IPR-treated group, incorporation of radioactivity into the trichloroacetic acid(TCA)-insoluble protein fraction was 42% higher than in the control group. The specific radioactivity of large mobile (LM) protein was 4.5-fold higher than that of the TCA-insoluble protein fraction. The stimulatory effect of IPR was markedly suppressed by the pretreatment of rats with actinomycin D. Actinomycin D inhibited the increase of LM protein concentration in submandibular glands by more than 90%, and markedly suppressed the incorporations of radioactivity into TCA-insoluble protein fraction and LM protein, indicating that the LM protein was not derived from other proteins, but was newly biosynthesized after IPR administration. The amount of LM protein in submandibular glands reached 27.6% of total soluble protein, and the steady-state level was achieved at the 11th day upon chronic IPR administration. The rate constant of disappearance of LM protein was estimated to be 0.23/d. The half-life and the rate constant for its biosynthesis were calculated to be 3.0 d and 410 $\mu g \cdot 100\ mg\ gland^{-1} \cdot d^{-1}$, respectively.

Keywords——submandibular gland; isoproterenol; hypertrophy and hyperplasia; saliva protein; LM protein; protein synthesis

Introduction

Isoproterenol (IPR), a β-adrenergic drug, is known to accelerate protein secretion from and to stimulate protein synthesis in the salivary glands of rats.[1-3] Menaker *et al.*[4] reported that a large mobile (LM) protein which was not observed in normal rats appeared in enlarged submandibular gland upon chronic IPR administration. We have shown that the LM protein which accumulated in the submandibular gland upon IPR treatment was secreted through the action of β-adrenergic receptors,[5] and the protein was formed in the gland when both phenoxybenzamine (an α-adrenergic receptor blocking drug) and adrenaline were administered together in rats.[6] However, the mechanism of its formation or accumulation in submandibular gland has not been fully clarified. In this paper, the half-life and the rate constants for biosynthesis and disappearance of LM protein were calculated. Experiments on *in vivo* (^{3}H)-lysine incorporation into the protein of submandibular glands were performed, in an attempt to determine whether the LM protein was newly biosynthesized or derived from other proteins upon IPR administration.

Materials and Methods

Male Sprague–Dawley rats (200—300 g weight) were kept as described previously.[5] *dl*-Isoproterenol-HCl (IPR) was from Nakarai Chemicals (Kyoto). Actinomycin D was a product of Sigma. L-(4,5-^{3}H)-lysine monohydrochloride (specific activity, 79.5 Ci/mmol) and a scintillant fluid (ACS II) were from the Radiochemical Centre, Amersham. Other reagents used were of special grade.

Incorporation of (^{3}H)-lysine into Proteins of Submandibular Gland——The animals in the experimental groups were injected intraperitoneally (*i.p.*) with 20 mg/kg of IPR once a day for two days. One of the experimental groups was injected *i.p.* with 200 μg/kg of actinomycin D 30 min before the first IPR injection. The control group received *i.p.* an equal volume of sterilized saline. On the third day, all animals were

injected *i.p.* with 200 μCi/kg of (^{3}H)-lysine. After (^{3}H)-lysine injection, the animals were sacrificed by exsanguination, and the submandibular glands were excised and frozen at −20°C. Soluble protein fraction (100000×*g* supernatant) from submandibular glands was prepared as described previously.[5] Separation o thef trichloroacetic acid (TCA)-insoluble protein fraction from the soluble protein fraction was carried out by the method of Schneider.[7] Separation of the LM protein from the soluble protein fraction was carried out by immunoprecipitation with a specific antibody prepared as described previously.[5] Antibody or control serum, 0.4 ml, was mixed with 0.2 ml of the soluble protein fraction and 0.4 ml of 0.001 M phosphate buffer (pH 7.2) containing 0.15 M NaCl (P.B.S.). The mixture was incubated for 1 h at 37°C and then was left standing at 4°C overnight. The precipitate was separated by centrifugation at 4000×*g* for 10 min. The resulting precipitate was washed three times with cold P.B.S. LM protein in the soluble protein fraction was purified on a diethylaminoethyl (DEAE)-cellulose column (1.2×24 cm) equilibrated with 0.01 M phosphate buffer, pH 7.2. An aliquot (10 ml) of the soluble protein fraction obtained from an IPR-treated group was loaded on the column and stepwise elution was carried out with the buffer containing 0, 0.25 M and 1.0 M NaCl. The eluted LM protein fraction was further purified by Sephadex G-75 column chromatography. The partially purified LM protein was analyzed by 11.25% polyacrylamide gel electrophoresis.[5] After electrophoresis, the gel was sliced into 1 mm sections. The gel slices were solubilized with 60% perchloric acid and 30% hydrogen peroxide according to the method of Manhin and Lofberg.[8] For counting of the radioactivities of the solubilized slices, fractions eluted from the DEAE-cellulose column and from the Sephadex G-75 column were mixed with 10 ml of ACS II. The other samples were completely digested and taken up in scintillant fluid by the use of an automatic combustion apparatus (Model ASC-111, Aloka Corp., Tokyo). The scintillant fluid consisted of 4 g of 2,5-diphenyloxazole, 0.4 g of 1,4-bis(2-(5-phenyloxazolyl))benzene and 100 g of naphthalene dissolved in 1 l of dioxane–toluene–methanol(7.5:1.5:1, v/v/v). Counting of radioactivity was performed with a scintillation counter (Model LSC-653, Aloka Corp. ,Tokyo).Thecounting efficiency was determined by the external standard ratio method.[9]

Measurement of the Rate Constant for Disappearance of LM Protein——The rats were injected *i.p.* with 20 mg/kg of IPR once a day from 1 to 13 da. On one day after the last IPR administration, the rats were anesthetized by injection of sodium pentobarbital (40 mg/kg of body weight, *i.p.*) and the right submandibular gland was excised. The rats were injected *i.p.* with 20 mg/kg of IPR as a secretory stimulant. The left submandibular gland was excised 90 min after the injection. Soluble protein fractions were prepared from both submandibular glands and the amount of LM protein was measured. A rate constant for disappearance of LM protein was estimated according to the method of Segal *et al.*[10] It was assumed that the rate of biosynthesis is independent of LM protein concentration in the gland and that the rate of disappearance is first order in LM protein concentration (equation 1).

$$\mathrm{d}(E)/\mathrm{d}t = k_1 - k_2(E) \tag{1}$$

In this equation, (E), t, k_1 and k_2 stand for LM protein concentration in the gland at a given time, time, the rate constant of biosynthesis and the rate constant of disappearance. At the steady state after IPR treatment, $\mathrm{d}(E)/\mathrm{d}t=0$, and therefore, equation (1) becomes:

$$k_1/k_2 = (E_0) \tag{2}$$

where (E_0) is the LM protein concentration at the steady state. Integration of equation (1) gives

$$\ln \frac{(k_1/k_2-(E))}{(k_1/k_2-(E_{\mathrm{init.}}))} = -k_2t \tag{3}$$

where $(E_{\mathrm{init.}})$ is the concentration of LM protein in normal rat submandibular gland. In this case, it is negligible. From equation (2) and equation (3), we obtain

$$\ln \frac{(E_0)-(E)}{(E_0)} = -k_2t \tag{4}$$

Equation (4) can be rearranged as follows:

$$\ln((E_0)-(E)) = \ln(E_0) - k_2t \tag{5}$$

For the calculation of k_2, the time before reaching the steady state was plotted against $\ln((E_0)-(E))$. When the concentration of LM protein (E) is one-half the concentration at the steady state (E_0), $(E)=(E_0)/2$. Under these conditions, equation (4) gives:

$$\ln 1/2 = -k_2t_{1/2}, \quad \text{or} \quad t_{1/2} = 0.693/k_2 \tag{6}$$

where $t_{1/2}$ stands for the half-life of the LM protein.

Protein Determination——Protein was determined by the method of Lowry *et al.*[11] using bovine serum

albumin as a standard. LM protein concentration was determined by a single radial immunodiffusion method.[5,12]

Results

The amount of antibody required for complete precipitation of LM protein in the soluble protein fraction obtained from submandibular glands was determined by titrating the LM protein against the antibody (Fig. 1). A solution of LM protein (350 μg/ml) was mixed with the antibody solution. After centrifugation, the amount of remaining LM protein in the supernatant fluid was estimated by a single radial immunodiffusion method as described previously.[5] From this experiment, 1 ml of antibody solution was calculated to be equivalent to 175 μg of the LM protein.

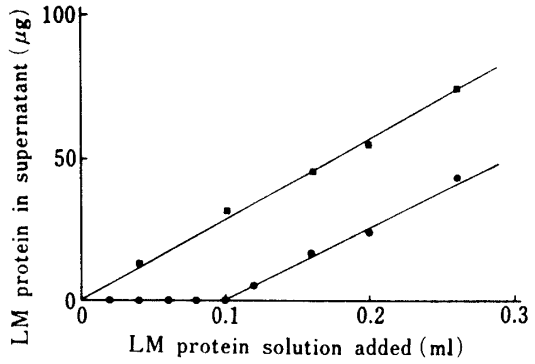

Fig. 1. Titration of Antibody and Control Serum with LM Protein

Antibody (●—●) or control serum (■—■) (0.2 ml) was mixed with LM protein (350 μg/ml) in 0.001 M phosphate buffer (pH 7.2)–0.15 M NaCl in a final volume of 0.6 ml. The mixture was incubated at 37°C for 1 h, and then was left standing at 4°C overnight. After centrifugation, appropriate aliquots of the supernatant solution were taken for the determination of LM protein by single radial immunodiffusion assay as described previously.[5]

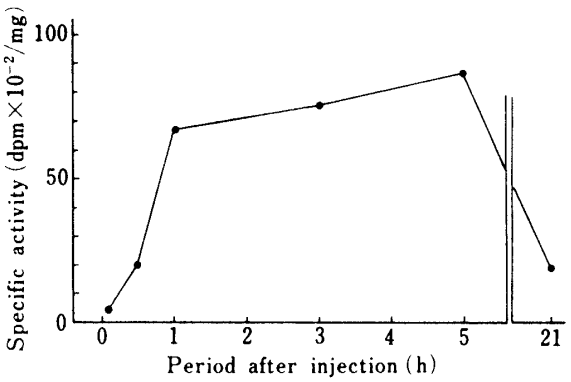

Fig. 2. Time Course of the *in Vivo* Incorporation of (^{3}H)-Lysine into the TCA-insoluble Protein Fraction of Submandibular Glands in IPR-treated Rats

Rats were injected with 20 mg/kg of IPR once daily for 2 days. One day after the last injection, the rats weregiven an injection of (^{3}H)-lysine (200 μCi/kg) then killed at the time indicated, and the radioactivity in the TCA-insoluble protein fraction was determined as described in the text.

The time course of (^{3}H)-lysine (200 μCi/kg) incorporation *in vivo* into TCA-insoluble protein fraction of submandibular glands was measured by using rats treated with 20 mg/kg of IPR for two days (Fig. 2). The specific radioactivity of TCA-insoluble protein increased linearly for 1 h after (^{3}H)-lysine injection, but increased only slightly in the following 4 h. At 21 h after the injection, however, only 25% of the radioactivity remained in the glands. During 1 h after the injection, 0.4% of the injected radioactivity was incorporated into the glands. The distributions of radioactivity in TCA-insoluble protein fraction, $100000\times g$ pellet (membrane fraction) and TCA-soluble fraction (including free (^{3}H)-lysine) were 10.5, 15 and 74.5%, respectively. The effects of IPR treatment and actinomycin D pretreatment on (^{3}H)-lysine incorporation into the TCA-insoluble protein fraction and LM protein were examined and the results are shown in Table 1. The amount of TCA-insoluble protein fraction obtained from soluble protein fraction of the gland homogenates was not increased significantly by IPR treatment. However, the radioactivity incorporated into this fraction increased 42% as compared to that of control group. The amount of TCA-insoluble protein fraction and radioactivity in this fraction decreased by 23.7 and 66.7%, respectively, on pretreatment with actinomycin D. On the other hand, the LM protein (which was not detectable in submandibular glands of the control group) was increased to 220 μg/100 mg gland by the IPR treatment. Pretreatment with actinomycin D reduced the amount of LM protein in the IPR-

treated group to 20 μg/100 mg gland. As compared to the control group, the IPR treatment increased the specific radioactivity of TCA-insoluble protein fraction (dpm/mg of protein) 1.4-fold. The specific activity of the LM protein was 4.5-fold higher than that of TCA-insoluble protein fraction.

TABLE I. Effect of Actinomycin D on the Incorporation of (^{3}H)-Lysine into Proteins in Submandibular Glands of IPR-treated Rats

		Control Mean ± S.E. (n=5)	IPR Mean ± S.E. (n=6)	Actinomycin D ± IPR Mean ± S.E. (n=6)
Soluble protein fraction	mg/100 mg	6.00±0.18	6.50±0.14	6.25±0.18
Submandibular gland	dpm/100 mg	125820±10520	176670±9980	101670±10430
TCA-insoluble protein fraction	mg/100 mg	3.71±0.16	3.72±0.11	2.84±0.20
Submandibular gland	dpm/100 mg	17600±2520	25000±3410	8330±1870
LM protein	μg/100 mg	0	220±32	20±7
Submandibular gland	dpm/100 mg	0	6610±130	180±4

Rats were injected with 20 mg/kg of IPR once daily for 2 d, while control rats were given an equal volume of saline. Actinomycin D (200 μg/kg) was injected 30 min before the first IPR injection. One day after the last injection, the rats were injected with 200 μCi/kg of (^{3}H)-lysine and glands were excised and weighed 1 h after the injection. The soluble protein fraction was prepared as described previously[5] and TCA-insoluble protein fraction was prepared according to the method of Schneider.[7]

The (^{3}H)-labeled LM protein was partially purified by chromatography on a DEAE-cellulose column and a Sephadex G-75 column (data not shown). On polyacrylamide gel electrophoresis of the partially purified LM protein (Fig. 3), the radioactivity peak and protein band coincided fairly well, indicating that (^{3}H)-lysine was really incorporated into the LM protein.

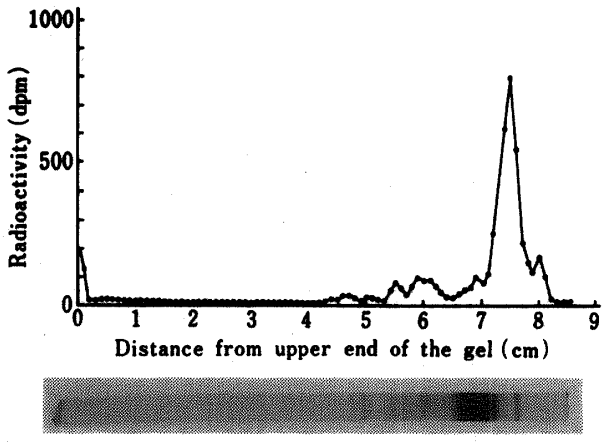

Fig. 3. Polyacrylamide Gel Electrophoresis of Partially Purified LM Protein labeled with (^{3}H)-Lysine

Partially purified LM protein (100 μg protein) was subjected to polyacrylamide gel electrophoresis and the radioactivities of the gel slices were deter mined as described in the text. Fifty μg of proteins was applied to the staining gel.

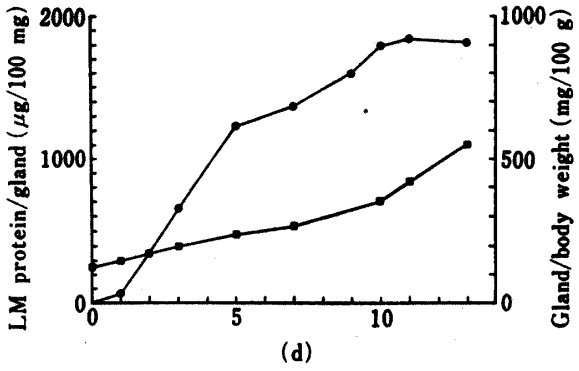

Fig. 4. Effect of IPR Administration on the LM Protein Content and the Wet Weight of Submandibular Glands

Rat were injected once daily for 1 to 13 d with 20 mg/kg of IPR, then killed 1 d after the last injection, and the weight of gland/body weight (mg/100 g) (■—■) was determined. The glands were then homogenized in 10 volumes of 0.01 M phosphate buffer (pH 7.2) in a glass-Teflon homogenizer. LM protein concentration (●—●) in the soluble protein fraction (100000× g supernatant fluid) was determined by a single radial immunodiffusion method.[5] Each point represents the mean of five separate determinations.

Chronic administration of IPR resulted in a gradual increase of LM protein in the glands, and a steady state was reached at the 11th day of IPR administration. The concentration of LM protein at the steady state (E_0) was 1822 μg/100 mg of submandibular glands (Fig. 4). As

previously reported,[5] from 70 to 80% of LM protein which accumulated in submandibular glands upon chronic administration of IPR was secreted from the glands upon IPR injection (data not shown). The increase in wet weight of the glands upon IPR treatment did not parallel the increase in the LM protein. On the 13th day after the initial IPR injection, the wet weight of the glands was about 5-fold more than that of the control group (Fig. 4). To determine the rate constant of disappearance of LM protein (k_2), the time before reaching the steady state level was plotted against ln $((E_0)-(E))$ (Fig. 5). k_2 was estimated to be 0.23/d. The half-life ($t_{1/2}$) and the rate constant for the biosynthesis of LM protein (k_1) were calculated as described in "Materials and Methods." $t_{1/2}$ and k_1 for this protein were calculated to be 3.0 d and 410 $\mu g \cdot 100$ mg gland$^{-1} \cdot d^{-1}$.

Discussion

The LM protein was not detectable in saliva secreted from submandibular glands of normal rats upon single IPR stimulation. However, a protein (protein A) with a relative mobility similar to that of LM protein on 11.25% polyacrylamide gel electrophoresis was present in the saliva.[5] Since the amount of protein A tended to decrease upon IPR treatment, it appeared that the LM protein was derived from protein A. However, (^{3}H)-lysine was effectively incorporated into the LM protein, the specific activity of the protein being 4.5-fold higher than that of TCA-insoluble protein fraction. Furthermore, the incorporation of radioactivity into LM protein was significantly inhibited by actinomycin D. These results clearly indicate that the LM protein is not a catabolite of preformed protein A, but is newly biosynthesized upon IPR treatment. Although the present experiments do not exclude the existence of precursors of the LM protein, protein A does not appear to a precursor, since anti-LM protein antibody did not react at all with protein A.

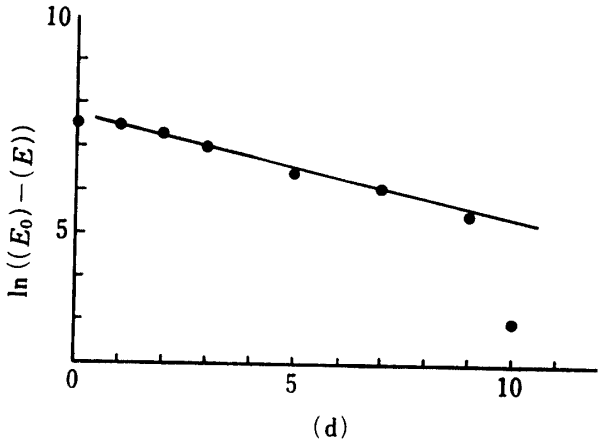

Fig. 5. Determination of Rate Constants

Data in Fig. 4 on LM protein content in submandibular glands were analyzed by means of the following equation: $\ln((E_0)-(E))=\ln(E_0)-k_2t$. In this equation, (E_0), (E), k_2 and t denote LM protein content in the steady state, LM protein content at a given time, the rate constant for disappearance and time, respectively.

The biochemical activity and physiological role of the LM protein have not yet been clarified. However, this protein may be involved in the development of submandibular glands, since Sheetz and Menaker reported that a protein which was assumed to be LM protein was found in normal submandibular glands of 6-day-old rats but not in that of 10-day-old rats.[13]

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References and Notes

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