Effect of Administration of Actinomycin D and Protein-Synthesis Inhibitors on the Binding of Prolactin to the Mammary Gland Prolactin Receptor in the Ovariectomized Mid-Pregnant Mouse

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Abstract In ovariectomized mid-pregnant mice, specific prolactin (PRL) binding to the mammary receptor began to increase around 16 h of ovariectomy. The effects of an inhibitor of transcription (actinomycin D) and of translation (cycloheximide) on the increase in PRL binding were examined *in vivo*. Actinomycin D which was administered between 0 and 12 h after ovariectomy inhibited the increase in PRL binding, while cycloheximide inhibited the increase by the injection after 16 h of ovariectomy. The mammary gland, collected at 24 or 36 h of ovariectomy, was incubated in the presence of actinomycin D, cycloheximide or puromycin *in vitro*. Although actinomycin D showed little or no effect on the amount of PRL binding, inhibitors of translation suppressed the binding significantly as compared with in the absence. It was concluded that the increase in PRL binding caused by ovariectomy was mediated by the initiation of the receptor synthesis. It was suggested that transcription for the receptor synthesis occurred shortly after ovariectomy.

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As pregnancy is sustained by high levels of serum progesterone, the progesterone withdrawal acts as the trigger of abortion. It has been shown that ovariectomy induces an increase in mammary prolactin (PRL) receptor and initiates lactose synthesis in the mouse¹⁾ and rat²⁾. The concentration of placental lactogen is very high from mid- to late pregnancy and disappears rapidly from circulation after parturition^{6,12,13)}. It has been interpreted that the apparent low capacity of PRL binding during pregnancy is caused by a masking effect of a large amount of placental lactogen⁵⁾. With the evidence that simultaneous removal

of adrenal glands inhibits the ovariectomy-induced increase in the PRL receptors^{2,3)}, it is also probable that the progesterone withdrawal initiates synthesis of the PRL receptor.

In this study, an inhibitor of transcription or translation was injected at different time after ovariectomy and the effect of the inhibitor on the PRL binding to mammary gland was examined. The serum concentration of lactogenic activity was also determined by a radioreceptor assay.

Materials and Methods

Hormones and chemicals : Ovine PRL (NIH-P-

S 15) was supplied by NIADDK, Bethesda, Md., USA. Actinomycin D, cycloheximide, and puromycin were from Wako Chemicals, Osaka. Bovine serum albumin (BSA) was from Seikagaku Kogyo, Tokyo. Medium 199 was from Difco Laboratories, Detroit, Mich., USA.

PRL binding- and radioreceptor assays: PRL was iodinated by the lactoperoxidase and hydrogen peroxide method¹⁰⁾. The specific radioactivity of ¹²⁵I-PRL used was about 2.6 MBq/ μ g.

The procedure of PRL binding assay has previously been described¹²⁾. The third thoracic mammary gland was cut into 0.5-mm thick slices by a tissue slicer (Hotta Rika, Tokyo). Three slices (10–15 mg) were incubated at 37°C for 2 h in 1 ml Medium 199–0.2% BSA (pH 7.4) containing ¹²⁵I-PRL (80,000–100,000 cpm) in the presence or absence of 5 μ g of unlabeled PRL. At the end of the incubation, the slices were washed with physiological saline and weighed. Radioactivity in the slices was determined in a gamma counter. The experiments were performed in duplicate.

The serum lactogenic activity was determined by a radioreceptor assay using the mid-lactating mammary gland and ovine PRL⁹⁾. Fifty μ l of the serum or unlabeled PRL solutions were included in the reaction mixture. The dose displacement curve was constructed using unlabeled PRL (0, 2.5–80 ng, 5 μ g) and the serum concentration of lactogenic activity was expressed as the concentration of standard ovine PRL.

In vivo and *in vitro* experiments: Inbred dd strain mice bred in our laboratory were used. The day on which a vaginal plug was found was taken as day 0 of pregnancy. Ovariectomy or sham-ovariectomy was performed under pentobarbitone sodium anaesthesia at 09.00–10.30 on day 13 of pregnancy. Animals were killed by cervical dislocation. Blood samples were collected by heart puncture without anaesthesia and the serum concentration of lactogenic hormone was measured by a radio-

receptor assay³⁾.

A single dose of actinomycin D (10 μ g/head) or cycloheximide (1 mg) dissolved in physiological saline was subcutaneously injected every 4 h from 0 to 24 h after ovariectomy. Animals were killed at 28 h of ovariectomy.

Mammary glands, collected at 24 and 36 h of ovariectomy, were incubated for 5 h at 37°C with actinomycin D (1 μ g/ml), cycloheximide (0.25–4 μ g/ml), or puromycin (10 μ g/ml) in the total volume of 1 ml. Incubation medium was Medium 199 containing 0.2% BSA (pH 7.4). Fifty μ l of the reaction mixture were removed at 3.5 h of the incubation, and 50 μ l of fresh medium containing ¹²⁵I-PRL with or without 5 μ g of unlabeled PRL were added.

Statistical analysis: The result is expressed as mean $\pm$ SE. Statistical analysis of the results was made by Student's two-tailed *t*-test. Differences were considered to be significant at $P < 0.05$.

Results

Changes in PRL binding and serum lactogenic activity after ovariectomy: The time-dependent changes in both specific ¹²⁵I-PRL binding to the mammary PRL receptor and serum concentration of lactogenic activity were examined in ovariectomized mice (Fig. 1). The amount of specific ¹²⁵I-PRL binding increased slightly during 16 h after ovariectomy, but increased rapidly thereafter, about 2.7 times at 20 h ($P < 0.05$) and reached maximum around 32 h. The amount at 32 h was about 7 times larger than that at 0 h or of sham-ovariectomized control ($P < 0.01$). Although the ovariectomized mice maintained the high level of lactogenic activity (140–212 ng/ml) in serum from 0 to 20 h of ovariectomy, the concentration decreased to about 18 ng/ml at 24 h of ovariectomy ($P < 0.01$). The low level was kept from 24 to 36 h. In the present experiments, abortion occurred around 20 h after ovariectomy.

Effects of actinomycin D and cycloheximide

Effect of Inhibitors on Mammary Prolactin Receptor

Fig. 1. Time-dependent changes in the PRL binding (○) and serum lactogenic activity (●) after ovariectomy. mean±SE, n = 5.

Fig. 2. a, b. Effects of actinomycin D and cycloheximide on the change in specific binding of PRL *in vivo*. (a) : Mice, received the injection of actinomycin D (closed) or cycloheximide (open) at the time after ovariectomy indicated, were used for the PRL binding assay at 28 h of ovariectomy. C : control injected with saline at 0 h of ovariectomy, S : sham-ovariectomized control. mean±SE, n = 5. Data were compared with the ovariectomized and saline-injected control. *; $P < 0.05$, **; $P < 0.01$. (b) : Cycloheximide was administered at 24 h of ovariectomy. Animals were killed at 6 and 12 h after the injection. Saline-injected (open) and cycloheximide-injected groups (closed). mean±SE, n = 4.

Fig. 3. Effects of actinomycin D, cycloheximide and puromycin on the change in specific binding of PRL *in vitro*. Mammary tissue slices, collected at 24 (open) or 36 h (closed) after ovariectomy, were incubated in the presence of actinomycin D (A), cycloheximide (C) or puromycin (P) for 5 h at 37°C. mean $\pm$ SE, n=5. Data were compared with that obtained in the absence of inhibitor. *; $P < 0.05$.

on PRL binding *in vivo*: Actinomycin D and cycloheximide were injected at different time after ovariectomy, and all mice were killed at 28 h of the operation. During 28 h, the amount of specific ^{125}I -PRL binding increased 6 times, compared with that at 0 h or of sham-ovariectomized control. As shown in Fig. 2a, actinomycin D and cycloheximide inhibited the increase in specific binding of ^{125}I -PRL in a different time-dependency. Actinomycin D was effective only when that was injected between 0 and 12 h of ovariectomy. By the injection at 4 h of ovariectomy, the amount of specific ^{125}I -PRL binding decreased to about half of that of the saline-injected control. The

effect of cycloheximide became effective by the injection after 16 h of ovariectomy. Regardless either actinomycin D or cycloheximide was injected and also regardless of the time of injection, the serum concentration of lactogenic activity did not differ from the saline-injected control and ranged in the low level from 15.5 to 19.0 ng/ml (data not shown). Fig. 2b showed that cycloheximide inhibited the increase in PRL binding until 6 h after the injection. The amount of ^{125}I -PRL, however, slightly increased by 12 h after injection. This suggests that cycloheximide is effective for less than 12 h after the injection *in vivo*.

Effects of actinomycin D, cycloheximide and

puromycin on PRL binding *in vitro*: The effect of inhibitors on PRL binding was examined in the *in vitro* system (Fig. 3). Although the addition of actinomycin D did not affect the amount of specific ^{125}I -PRL binding to the mammary tissue slices, both cycloheximide and puromycin decreased it significantly. Cycloheximide inhibited the binding in a dose dependent manner. At $4\text{ }\mu\text{g/ml}$, it decreased the amount of specific binding to 59% as compared with in the absence of inhibitor. The *in vitro* experiment also revealed that the mammary glands taken at 24 h after ovariectomy were more sensitive to cycloheximide and puromycin than those at 36 h.

Discussion

We have reported that the level of lactogenic activity in serum of mice at mid-pregnancy is very high¹²⁾, and the fact was confirmed in this study. The author showed here that this activity fell sharply between 20 and 24 h after ovariectomy, accompanied by the occurrence of abortion. As the serum concentration of PRL was kept unchanged between 0 and 24 h of ovariectomy³⁾, it is apparent that the placental lactogen disappears from the circulation after 20 h of ovariectomy. Therefore, it was possible to ascribe the increase in the binding of PRL to the mammary receptor to that the receptor became free of the masking effect of placental lactogen and then was able to bind with PRL. However, although the serum lactogenic activity had decreased to the basal level also in inhibitors-treated mice, both actinomycin D and cycloheximide inhibited the increase in PRL binding *in vivo*. In addition, cycloheximide and puromycin directly acted on the mammary gland and inhibited the increase in PRL binding in the *in vitro* experiment. The binding of PRL to the rat liver PRL receptor was the highest during pregnancy in spite of the high level of serum lactogenic activity⁴⁾. These evidence suggested that the increase in the PRL binding caused by ovariectomy is

ascribed to the newly synthesis of the receptor triggered by ovariectomy, rather than to the disappearance of the effect of placental hormones. The PRL binding increases in the presence of glucocorticoid hormones *in vivo*³⁾ and *in vitro*¹¹⁾. Until now, it is not known when synthesis of the PRL receptor is initiated by ovariectomy.

The effective period of an inhibitor of translation, cycloheximide, was later than that of an inhibitor of transcription, actinomycin D. The action of cycloheximide became apparent 16 h after ovariectomy, while the action of actinomycin D could be observed only when the inhibitor was given shortly after ovariectomy. This indicated that the initiation of mRNA synthesis for the receptor increase was induced shortly after ovariectomy. However, the inhibitory action of actinomycin D could no longer be observed after 16 h of ovariectomy. I speculated that the messenger RNA for the receptor synthesis had already been accumulated in the mammary gland by this time. This speculation would be supported by OHSAKO *et al.* who reported in the mid-pregnant rat that the rate of uridine incorporation into the mammary gland was very high between 0 and 16 h after ovariectomy⁸⁾, and that the lactose synthesis was suppressed by progesterone only when the hormone was administered within 4 h after ovariectomy⁹⁾.

In the present experiments, the time-dependent effect of an inhibitor of transcription or of translation on the binding of PRL to the mammary gland was clear in the ovariectomized mid-pregnant mouse. It was concluded that the increase in PRL binding caused by ovariectomy was mediated by the initiation of the receptor synthesis. It was suggested that transcription for the receptor synthesis occurred shortly after ovariectomy.

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妊娠中期卵巣除去マウス乳腺におけるプロラクチンレセ
プターへのプロラクチンの結合に及ぼすアクチノ
マイシン D と蛋白質合成阻害剤の影響

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卵巣除去した妊娠中期のマウスでは, 卵巣除去後 16 時間から乳腺へのプロラクチン結合量の増加が起る. この増加に対する転写阻害剤及び翻訳阻害剤の影響を調べた. インビボ実験においてアクチノマイシン D 及びサイクロヘキシミドの有効な効果がみられたが, 卵巣除去後の時間は両者と異なった. アクチノマイシン D は, 卵巣除去直後から 12 時間までに投与すると増加を抑制した. 一方, サイクロヘキシミドは, 除去後 16 時間以降に投与すると増加を抑制した. 次に卵巣除去後 24 時間及び 36 時間経過したマウスから得た乳腺組織をアクチノマイシン D, サイクロヘキシミド, あるいはピューロマイシンの存在下でインビトロで培養し, そのプロラクチン結合量に対する影響を調べた. アクチノマイシン D の添加はプロラクチン結合に影響を与えなかったが, サイクロヘキシミドおよびピューロマイシンは共に有意に結合を抑制した. これらの結果は, 卵巣除去によって新しくプロラクチンレセプターが作られること, 卵巣除去後早い時期から乳腺細胞でプロラクチンレセプターが作られるための転写が始まること, を示した.

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