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Gonadotropin-Dependent Expression of Sterol 14-Demethylase P450 (CYP51) in Rat Ovaries and Its Contribution to the Production of a Meiosis-Activating Steroid

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Immunohistochemical analysis showed that sterol 14-demethylase P450 (CYP51) is expressed in mature follicles and corpus lutea of rat ovaries. In follicles, CYP51 is expressed in granulosa and theca cells but not in oocytes. The ovarian CYP51 activity of hypophysectomized rats is very low and induced by pregnant mares' serum gonadotropin (PMSG) treatment together with ovarian growth. The expression of CYP51 first increases in growing follicles and then appears in the corpus lutea after luteinization. The former event may be due to the follicular-stimulating hormone action of PMSG, and the latter may be caused by the luteinizing hormone effect of PMSG. Sterol analysis indicated that the product of the CYP51-mediated lanosterol 14-demethylation, 4,4-dimethylcholesta-8,14,24-trienol, which has been identified as a meiosis-activating steroid (MAS) in mammals [Byskov *et al.* (1995) *Nature* 374, 559–562], accumulates (about 10 pmol/mg of ovary) in mature rat ovaries, and the content is enough to activate the resumption of meiosis. These lines of evidence suggest that the expression of ovarian CYP51 is dependent on gonadotropins, and ovarian CYP51 activity is enough for accumulating MAS. Serum insulin does not affect the ovarian CYP51 level, although it is essential for hepatic CYP51 expression. These findings indicate that expression of CYP51 is regulated differently among organs.

Key words: CYP51, cytochrome P450, gonadotropin, meiosis-activating steroid, ovary.

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Sterol 14-demethylase P450 (CYP51) is the enzyme catalyzing the 14-demethylation of sterol precursors such as lanosterol (*1*). CYP51 is the only known P450 enzyme present in all biological kingdoms (*2–4*). In mammals, CYP51 is ubiquitously expressed in most organs and tissues (*5, 6*), and the mammalian *CYP51* gene has the characteristics of a housekeeping gene (*5, 6*). These facts indicate that CYP51 and the 14-demethylation of sterol precursors catalyzed by this P450 are indispensable for maintaining eukaryotic life. Since sterols are essential compounds in eukaryotes, the fundamental importance of CYP51 is readily understood. However, many questions remain concerning mammalian CYP51, especially the regulation of its expression level in different organs as related to its function in each organ. The level of CYP51 expression varies widely among organs such as liver, testis, ovary, adrenal grand, intestine, spleen and brain, in terms of both mRNA level (*5, 6*) and activity (Yamashita *et al.*, unpublished), suggesting that CYP51 expression may be regulated in an organ-specific manner.

In the previous paper (*7*), we revealed that the expression of hepatic CYP51 is dependent on serum insulin level, and suggested the contribution of a sterol-regulatory element binding protein (SREBP)–1c to this regulation as in the case of other enzymes participating in lipid biosynthesis, such as HMG-CoA reductase and fatty acid metabolizing enzymes (*8*). The high expression of hepatic CYP51 depending on the above regulatory mechanism is quite reasonable since liver is the principal organ of cholesterol production. On the other hand, Rozman and coworkers (*9, 10*) presented evidence indicating that the expression of CYP51 in postmeiotic spermatids depends on CREMτ (cAMP-responsive element modulator protein τ). The size of the CYP51 mRNA produced in testis is smaller than in other organs (*5, 6, 10*). These facts suggest that CYP51 in male germ cells has its own regulatory mechanism related to its specific function in spermatogenesis.

In 1995, Byskov *et al.* (*11*) found that 14-demethylated derivatives of lanosterol act as meiosis-activating steroids (MAS) in mouse oocytes. Since CYP51 is the only known enzyme responsible for sterol 14-demethylation (*1*), MAS formation is a highly possible candidate for the organ-specific function of ovarian CYP51. The ovarian CYP51 expres-

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Abbreviations: CRE, cAMP-responsive element; CREM, cAMP-responsive element modulator protein; CYP51 and *CYP51*, sterol 14-demethylase P450 and its gene; FF-MAS, follicular fluid meiosis-activating steroid; FSH, follicular stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; LXR, liver sterol-X receptor; MAS, meiosis-activating steroid; PMSG, pregnant mares' serum gonadotropin; RXR, retinoid-X receptor; SRE, sterol-regulatory element; SREBP, sterol-regulatory element binding protein; T-MAS, testicular meiosis-activating steroid.

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sion level in prepubertal rats is low, and can be induced by gonadotropin treatment (*12*), suggesting a contribution of CYP51 to the function of the mature ovary. Byskov *et al.* (*11*) identified two naturally occurring MAS, 4,4-dimethylcholesta-8,14,24-trienol isolated from human follicular fluid and 4,4-dimethylcholesta-8,24-dienol obtained from bull testes, named FF-MAS and T-MAS, respectively. In the sterol biosynthetic pathway, FF-MAS is the direct metabolite of lanosterol by CYP51 (*1*) and T-MAS is formed from FF-MAS by sterol 14-reductase (*13, 14*). Accordingly, the amount of MAS accumulated in ovaries may be dependent on the relative expression levels of CYP51 and sterol 14-reductase to other enzymes in the cholesterol-synthesizing pathway. This is an interesting subject for studying the regulatory mechanism of CYP51 expression level as related to its organ-specific function. However, little is known about ovarian CYP51. This paper describes the results obtained in basic studies of the hormonal regulation of ovarian CYP51 expression. The results indicate that CYP51 is expressed in mature follicles and corpus lutea depending on pituitary gonadotropins. The results also reveal sufficient amounts of FF-MAS to activate the resumption of meiosis are present in mature rat ovaries, indicating that the ovarian CYP51 level is high enough for to allow the accumulation of FF-MAS, an intermediate in sterol biosynthesis.

MATERIALS AND METHODS

Experimental Animals and Treatments—All rats used in this study were maintained on a 12 h light and 12 h dark cycle and fed *ad libitum* normal laboratory rat chow and tap water. Two month-old female Wistar Imamichi rats (Japan SLC) with steady 4-day sexual cycles were used to analyze the estrus cycle-dependent variation in ovarian CYP51 levels. The estrus cycles of individual animals were monitored by microscopic examination of vaginal smears. Intact and hypophysectomized female Sprague-Dawley (SD) rats (3 to 6 weeks-old and 56–70 g in body weight) were also obtained from Japan SLC, and injected subcutaneously with 25 units of pregnant mares' serum gonadotropin (PMSG, Sigma). The PMSG-treated rats were sacrificed 24, 48, and 96 h after the injection. Hypophysectomized rats treated with PMSG for 48 h as above were injected subcutaneously with 10 units of human chorionic gonadotropin (hCG, Sigma), and sacrificed on the 1st, 3rd, and 7th day of hCG treatment. Two-month-old female SD rats were injected subcutaneously with streptozotocin (Sigma) dissolved in 0.1 M citrate buffer, pH 4.6, at a dose of 65 mg/kg of body weight, and sacrificed 18, 24, and 48 h after streptozotocin administration.

Preparation of Enzyme Source and Assay of CYP51 Activity—All animals were sacrificed by decapitation, and the ovaries were removed quickly. The pair of ovaries isolated from each rat were combined and homogenized with sonication in 0.1 M potassium phosphate buffer, pH 7.5. The ovarian homogenates were centrifuged at 10,000 ×*g* for 10 min, and the resulting supernatant served as the ovarian S-10 fraction for assaying ovarian CYP51 activity. Liver microsomes were prepared as described in the previous paper (*7*). CYP51 activity was assayed essentially by the same method as described previously (*12, 15, 16*). The reaction mixture for determining ovarian CYP51 activity consisted of lanosterol (23.5 nmol, dispersed in Tween 80), S-10 fraction (2–3 mg protein), KCN (1 mM), an NADPH-generating system (0.15 mM NADPH, 10 mM glucose 6-phosphate, and 0.2 u glucose-6-phosphate dehydrogenase) and 0.1 M potassium phosphate buffer, pH 7.5, in a final volume of 2.0 ml. The reaction was carried out aerobically at 37°C for 30 min with constant shaking. The reaction was terminated by saponification, and the sterols were extracted with diethyl ether/petroleum ether (5/95). The extracted sterols were separated by TLC (*15*), and the fractions containing lanosterol and its 14-demethylated metabolites were extracted (*15*). The extracted sterols were trimethylsilylated and analyzed by GLC (*15*). The demethylase activity was calculated from the chromatographically determined conversion ratio of lanosterol to the two demethylated metabolites (*15–17*) and the initial amount of lanosterol after correction for endogenous metabolites by gas-chromatography of sterols extracted from the reaction mixture at time zero of incubation.

Immunohistochemistry—Ovaries were embedded in Tissue-Tek® O.C.T. 4583 compound (Sakura Finetek USA) and frozen at –70°C on dry ice-acetone. The frozen blocks were sliced into 8 μm sections at –20°C with a TISSUE-TEK II cryostat microtome (Miles-Sankyo), and the sections were mounted on ovalbumin-coated slide glasses. The immunohistochemical detection of ovarian CYP51 was done using anti-rat CYP51 antibodies as the primary antibodies and a Pathostain® ABC-POD kit (Wako Pure Chemicals). Immunostaining was performed according to the instruction manual for the reagent kit. The anti–rat CYP51 antibodies were prepared from the serum of a rabbit immunized with the purified rat CYP51 expressed in *Escherichia coli* (*18*). The antibodies gave a single immunopositive band corresponding to CYP51 upon western blotting of the microsomal proteins from liver, adrenal grand, testis, ovary, and brain, and a good correlation was obtained between the immunochemically determined CYP51 contents and the CYP51 activity of each tissue (Aoyama *et al.*, unpublished). These results indicate that the antibodies show practically no cross-reaction with other P450 species in these tissues.

Other Methods—Ovarian and hepatic CYP51 mRNA levels were determined by northern blot analysis (*7*). RNA was prepared using TRIzol® reagent (Life Technologies) according to the instruction manual for the reagent. The sterol content of rat ovaries was determined by the same method used to determine the products of the 14-demethylation reaction (see above), except that an ovarian homogenate was used as the starting material. Serum insulin was determined using a Glazyme® insulin immunoassay kit (Wako Pure Chemicals). Protein was determined by the method of Lowry *et al.* (*19*) using bovine serum albumin as the standard.

RESULTS

Ovarian CYP51 Activity and Sterol Content in Mature Rats—Ovarian CYP51 activities of Wistar-Imamichi rats showing a steady 4-day estrus cycle were analyzed in relation to estrus cycle. As shown in Fig. 1, ovarian CYP51 activity is apparently high in diestrus (9.96 ± 3.47 pmol/min/mg of S-10 protein) and proestrus (8.98 ± 4.21 pmol/min/mg of S-10 protein). The ovarian CYP51 activity in these stages showed considerable variation and the difference in the CYP51 activities between these two and the other three

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stages was not statistically significant. However, high ovarian CYP51 activities (>12 pmol/min/mg of S-10 protein) were found only same rats in diestrus and proestrus. Since the final growth of follicles prior to ovulation occurs in diestrus and proestrus, the above results suggest that CYP51 activity may increase in follicles in the final stage of maturation prior to ovulation.

Figure 2 represents typical immunohistochemical staining of a diestrus rat ovary. Rats have an incomplete estrus cycle and the ovaries of diestrus rats contain maturing follicles and corpus lutea. CYP51 immunostaining was observed in the follicles (Fig. 2A) and corpus lutea (Fig. 2B). In the follicles, CYP51 expression was observed in theca, granulosa, and cumulus cells, but not in oocytes (Fig. 2A). The intensity of CYP51 immunostaining was higher in theca cells than in granulosa and cumulus cells (Fig. 2A). The intensity of the immunostaining was somewhat higher in the cumulus and the medial granulosa cells than in the peripheral granulosa cells (Fig. 2A), and this phenomenon was observed in many mature follicles.

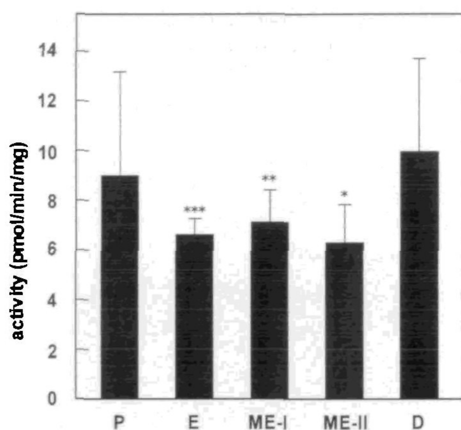
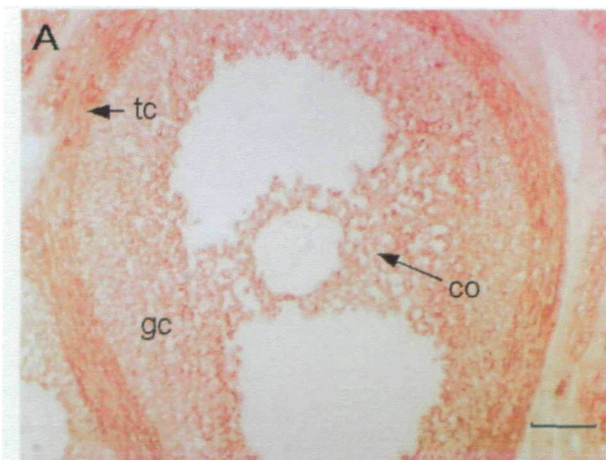


Fig. 1. **Ovarian CYP51 activities of rats in different estrus stages.** The estrus stages of two-month-old female rats were evaluated by microscopic examination of vaginal smears, and ovarian CYP51 activity of individual rats was determined. P, E, ME-I, ME-II, and D denote proestrus, estrus, metestrus-I, metestrus-II, and diestrus, respectively. Vertical bars indicate standard deviations and *, **, and *** denote $p < 0.005$, $p < 0.05$, and $p < 0.1$, respectively relative to D. The number of rats used in each experiments were 13, 4, 10, 12, and 36 for P, E, ME-I, ME-II, and D, respectively.

Table I shows the contents of lanosterol and FF- and T-MAS in mature rat ovaries. The content of FF-MAS is considerably higher than those of lanosterol and T-MAS. FF-MAS is an intermediate of cholesterol biosynthesis derived from lanosterol by CYP51 (*1*) and is converted to T-MAS by sterol 14-reductase (*13, 14*). Therefore, ovarian CYP51 activity (6–12 pmol/min/mg S-10 protein) might be high enough to maintain the ovarian FF-MAS level (10.4 pmol/mg wet tissue). Assuming that the relative density of the ovary is nearly one, the apparent concentration of FF-MAS in the ovary is calculated to be about 10 μM, and this concentration is high enough to induce the resumption of meiosis *in vitro* (*11*). It is also noteworthy that the standard deviations for the FF-MAS and T-MAS levels are significantly greater than that of lanosterol, suggesting that the amount of FF- and T-MAS accumulated in ovaries varies considerably among individuals; this may be related to the variation in CYP51 activity during the estrus cycle as shown in Fig. 1.

TABLE I. **Lanosterol, FF-MAS, and T-MAS contents of mature rat ovaries.** Ovaries isolated from one mature rat were sonicated in 0.1 M potassium phosphate buffer, pH 7.5. Sterols were extracted from the homogenate after saponification and analyzed by GLC by the same method used for the assay of CYP51 activity as described in "MATERIALS AND METHODS." The amounts of lanosterol, FF-MAS, and T-MAS were calculated from their peak areas using the calibration curve obtained with known amounts of lanosterol. Values are the mean ± standard deviation of 6 independent experiments.

Sterols	Contents
	pmol/a couple of ovaries
Lanosterol	271 ± 21
FF-MAS	642 ± 142
T-MAS	160 ± 45



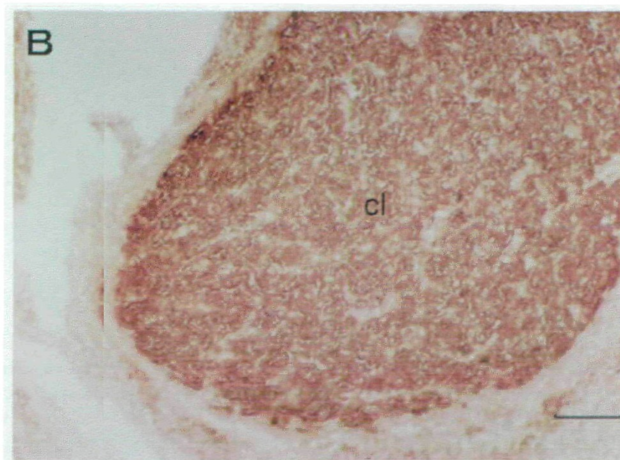


Fig. 2. **Immunohistochemical detection of CYP51 expressed in a mature rat ovary.** Frozen ovarian slices (8 μm) were immunostained as described in "MATERIALS AND METHODS" using anti-rat CYP51 antibodies as the primary antibodies. Positive CYP51 immunostaining was observed on cumulus (co), granulosa (gc), and theca (tc) cells of a follicle (Plate A) and a corpus luteum (cl) (Plate B). Note that the intensity of immunostaining is somewhat stronger in the cumulus and medial granulosa cells than in the peripheral granulosa cells (Plate A). Black bar = 100 μm.

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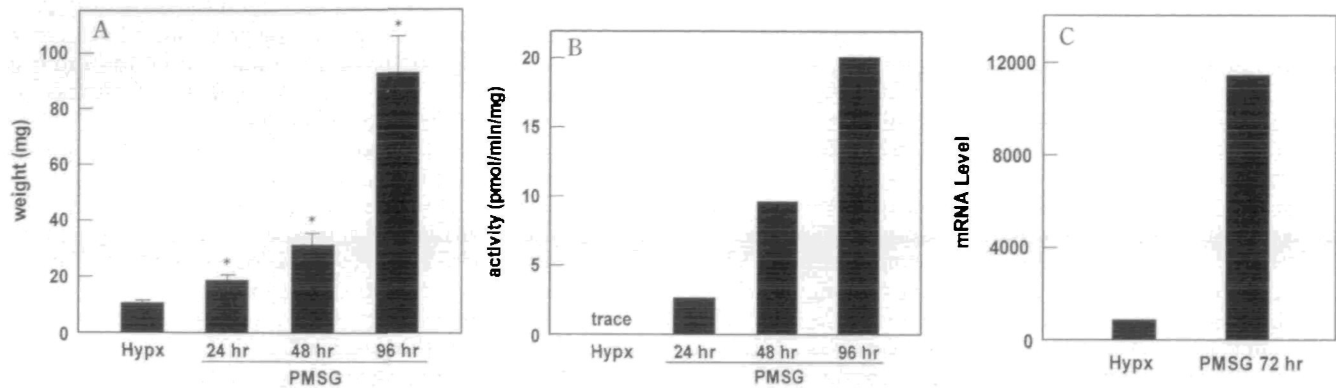

Fig. 3. **Effects of PMSG on ovarian weight, CYP51 activity and CYP51 mRNA levels of hypophysectomized rats.** PMSG (25 IU in 0.1 ml saline) was administered subcutaneously to hypophysectomized rat (4-weeks old). The rats were sacrificed 24, 48, and 96 h after treatment. Panel A: the mean and standard deviation of ovarian weight of six rats (* denotes $p < 0.001$). Panel B: the mean of ovarian CYP51 activity. Ovarian S-10 was prepared from the combined ovaries of six rats in each experiment. Panel C: CYP51 mRNA levels determined by northern blot analysis. Poly(A)$^+$ RNA was prepared from the ovaries of five hypophysectomized and two PMSG-treated rats. mRNA levels are expressed in arbitrary units.

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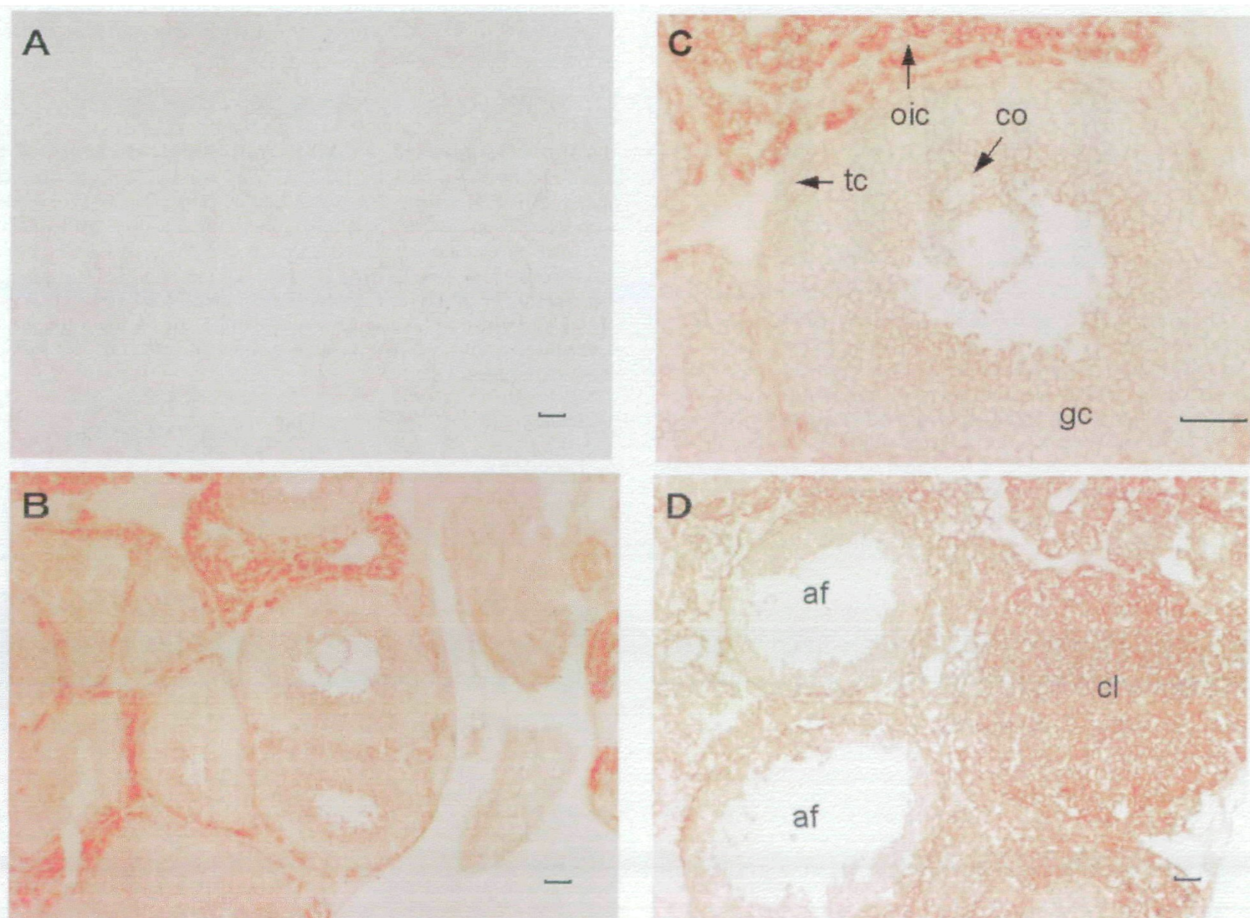

Fig. 4. **Immunohistochemical detection of CYP51 expressed in the ovaries of hypophysectomized and the PMSG-treated hypophysectomized rats.** Frozen ovarian slices (8 μm) were immunostained as described in "MATERIALS AND METHODS" using anti-rat CYP51 antibodies as the primary antibodies. Plate A: an ovary of a hypophysectomized rat. Plate B: an ovary of a PMSG-treated hypophysectomized rat isolated on the 2nd day of PMSG treatment. Plate C: magnified view of part of plate B. Plate D: an ovary of a PMSG-treated hypophysectomized rat isolated on the 4th day of PMSG treatment. Immunostaining of CYP51 in the hypophysectomized rat is very weak and detected only on the surface area of immature follicles (Plate A). In ovaries of PMSG-treated rats (Plates B, C, and D), positive CYP51 immunostaining is observed on the cumulus (co), granulosa (gc), and theca (tc) cells of the follicles, corpus lutea (cl), and cavitated atretic follicles (af). The higher expression of CYP51 in the cumulus and medial granulosa cells than in peripheral granulosa cells, as pointed out in Fig. 2A, is more apparent in this specimen (Plates B and C). High CYP51 expression can be observed also on ovarian interstitial cells (oic). Black bar = 100 μm.

Effects of Hypophysectomy and Gonadotropin Treatment of Hypophysectomized Rats on Ovarian CYP51—In the previous paper (*12*), we demonstrated that the ovarian CYP51 activity in immature rat is induced by PMSG. To obtain further information about the role of gonadotropins in the regulation of ovarian CYP51 expression, the effects of hypophysectomy and PMSG administration on ovarian CYP51 were studied. Hypophysectomized rats (4 weeks old and 1 week after surgery) showed dwarf characteristics, and the average ovary weight was 10.5 ± 0.8 mg, about one third the size in normal 4-week-old rats. The ovary weight of the hypophysectomized rats increased markedly following a single administration of PMSG, to nearly 9-fold on the 4th day after treatment (Fig. 3A). Ovarian CYP51 activity in hypophysectomized rats was very low close to the detection limit of the assay (Fig. 3B). The administration of PMSG induced ovarian CYP51 activity to 9.5 pmol/min/mg S-10 protein 48 h after PMSG treatment (Fig. 3B). This activity is comparable to that of normal rats, and the activity increased further until the 4th day after treatment (Fig. 3B). The effects of hypophysectomy and PMSG treatment on the ovarian CYP51 mRNA level were evaluated by northern blotting in a separate experiment. The suppressed ovarian CYP51 mRNA level in hypophysectomized rats increased markedly upon PMSG administration (Fig. 3C). These results indicate the necessity of pituitary gonadotropins for the expression of ovarian CYP51, and that the induction of ovarian CYP51 by PMSG occurs at the transcriptional level.

Figure 4 shows the immunohistochemical staining of ovarian CYP51 in a hypophysectomized rat. As shown in Fig. 4A, the ovaries of hypophysectomized rats contain small immature follicles, and weak CYP51 immunostaining was detected only in the surface area of these follicles. PMSG administration caused marked growth of the follicles 24 h after injection, and many follices reached nearly mature size on the 2nd day after PMSG treatment. The intensity of CYP51 immunostaining increased in both granulosa and theca cells (Fig. 4, B and C). As pointed out in the preceding section (Fig. 2A), the intensity of CYP51 immunostaining was somewhat higher in the cumulus and medial granulosa cells than in peripheral granulosa cells. This phenomenon was also observed in the PMSG-induced follicles of hypophysectomized rats, and the difference between the intensities of immunostaining of the medial and peripheral parts of the granulosa was greater in these follicles (Fig. 4, B and C) than in those of normal rats (Fig. 2A). Marked CYP51 immunostaining was observed on ovarian interstitial cells (Fig. 4, B and C), although this was not observed in normal mature rat ovaries. On the 4th day after PMSG treatment, the ovaries were enlarged and the follicles were apparently luteinized. CYP51 immunostaining was observed in the corpus lutea, and the CYP51 expression level apparently increased upon luteinization (Fig. 4D). These immunohistochemical observations suggest that CYP51 is expressed slightly in immature follicles in hypophysectomized rats and is markedly induced upon PMSG administration to the rats. This induction of CYP51 activity by PMSG (Fig. 3B) is due to the synergism of follicular growth and the elevation of the follicular CYP51 level, which may be due to the FSH action of PMSG. Luteinization and CYP51 expression in the corpus lutea occurring two days after PMSG treatment caused a further induction of CYP51 (Fig. 3B), which may be due to the LH action of PMSG.

Effects of hCG Treatment of PMSG-Treated Hypophysectomized Rats on the Ovarian CYP51 Expression—The expression of ovarian CYP51 increases upon luteinization. Ovulation and luteinization are initiated by the LH surge.

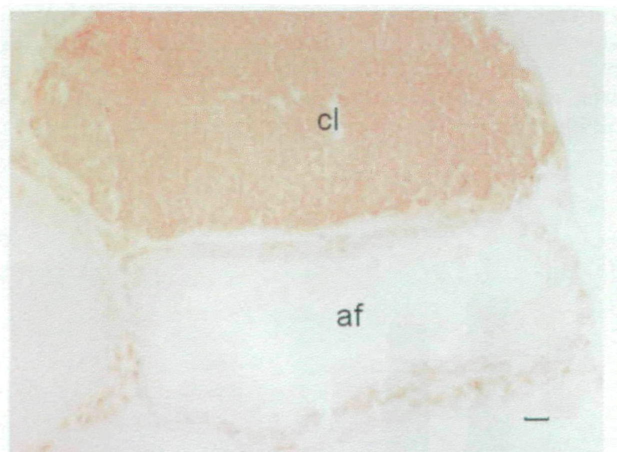

Fig. 6. **Immunohistochemical detection of CYP51 expressed in an ovary isolated on the 7th day of hCG administration to a PMSG-treated hypophysectomized rat.** CYP51 immunostaining is observed on corpus lutea (cl), but has almost disappeared from the cavitated atretic follicles (af). Black bar = 100 μm.

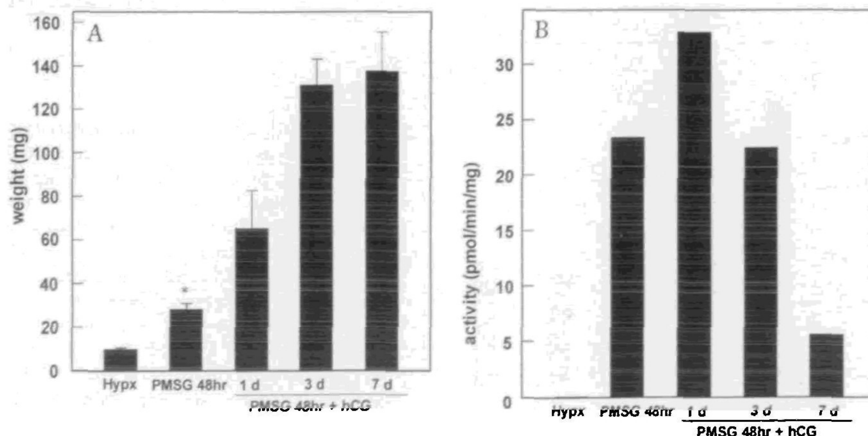

Fig. 5. **Effects of hCG administration on the weight and CYP51 activity of PMSG pretreated hypophysectomized rat ovaries.** PMSG (25 IU in 0.1 ml saline) was administered subcutaneously to hypophysectomized rat (4 weeks old). Fourty-eight hours after PMSG treatment, hCG was administered subcutaneously. The rats were sacrificed on the 1st, 3rd, and 7th day of hCG treatment. Panel A: weight of ovary with standard deviations. Panel B: ovarian CYP51 activity. Ovarian S-10 was prepared from the combined ovaries of three rats.

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Thus, the effects of hCG administration to PMSG-pretreated hypophysectomized rats on ovarian CYP51 expression were studied. PMSG was administered once to hypophysectomized rats (4 weeks old, 1 week after surgery) to induce follicular growth. At 48 h after PMSG treatment, hCG was administered, and the effects of hCG on ovarian CYP51 activity were examined on the 1st, 3rd, and 7th day after hCG treatment. The ovary weight increased by PMSG pretreatment and reached a plateau on the 5th day after PMSG treatment, which was the 3rd day after hCG treatment, and this level was maintained until the end of the experimental period (Fig. 5A). This plateau level was more than twice the average weight of mature rat ovaries. Ovarian CYP51 activity in hypophysectomized rats was induced by PMSG pretreatment and reached 23.5 pmol/min/mg S-10 protein before hCG injection (Fig. 5B). The administration of hCG to PMSG-pretreated rats resulted in a further elevation of the CYP51 activity to more than 30 pmol/min/mg S-10 protein 24 h after administration, but the activity decreased to 5 pmol/min/mg S-10 protein on the 7th day after hCG treatment (Fig. 5B). In the previous paper (*12*), we reported that the PMSG-induced CYP51 activity in immature rat ovaries begins to decrease on the 3rd day after treatment. Therefore, the elevation of CYP51 activity observed 24 h after hCG administration followed by a reduction in activity seems to suggest a transient elevation of the CYP51 level due to the LH action of hCG. Figure 6 shows the CYP51 immunostaining of ovaries isolated seven days after hCG treatment. CYP51 expression was still observed in the corpus lutea, however, the number of corpus lutea was decreased and little CYP51 expression was observed in the cavitated atretic follicles. This immunohistochemical observation is consistent with the decrease in ovarian CYP51 activity on the 7th day after hCG treatment (Fig. 5B).

Effect of Streptozotocin Treatment on Hepatic and Ovarian CYP51 Activity—We reported in the previous paper (*7*) that the expression of testicular CYP51 is not reduced in streptozotocin-induced diabetic rats, whereas the expression of hepatic CYP51 is maintained by serum insulin and diminished in streptozotocin-induced diabetic rats. Then, the effects of streptozotocin treatment on the expression of ovarian CYP51 were studied. Streptozotocin was injected one time subcutaneously into mature female SD rats (65 mg/kg of body weight), and the ovarian and hepatic CYP51 activities were analyzed 18, 24, 48, and 72 h after treatment. Serum insulin concentration decreased to about 30% of the normal value 24 h after streptozotocin administration, and decreased further to less than 20% of the normal value 72 h after treatment (Fig. 7A). Hepatic CYP51 activity and mRNA levels also decreased in response to the decreasing serum insulin concentration (Fig. 7B), as in the case of the previous experiments on male rats (*7*). However, no significant correlation was observed between the activity and mRNA levels of ovarian CYP51 (Fig. 7C) and the serum insulin concentration (Fig. 7A). These observations indicate that ovarian CYP51 expression is not susceptible to the decrease in serum insulin concentration that causes a marked reduction in the hepatic CYP51 expression level.

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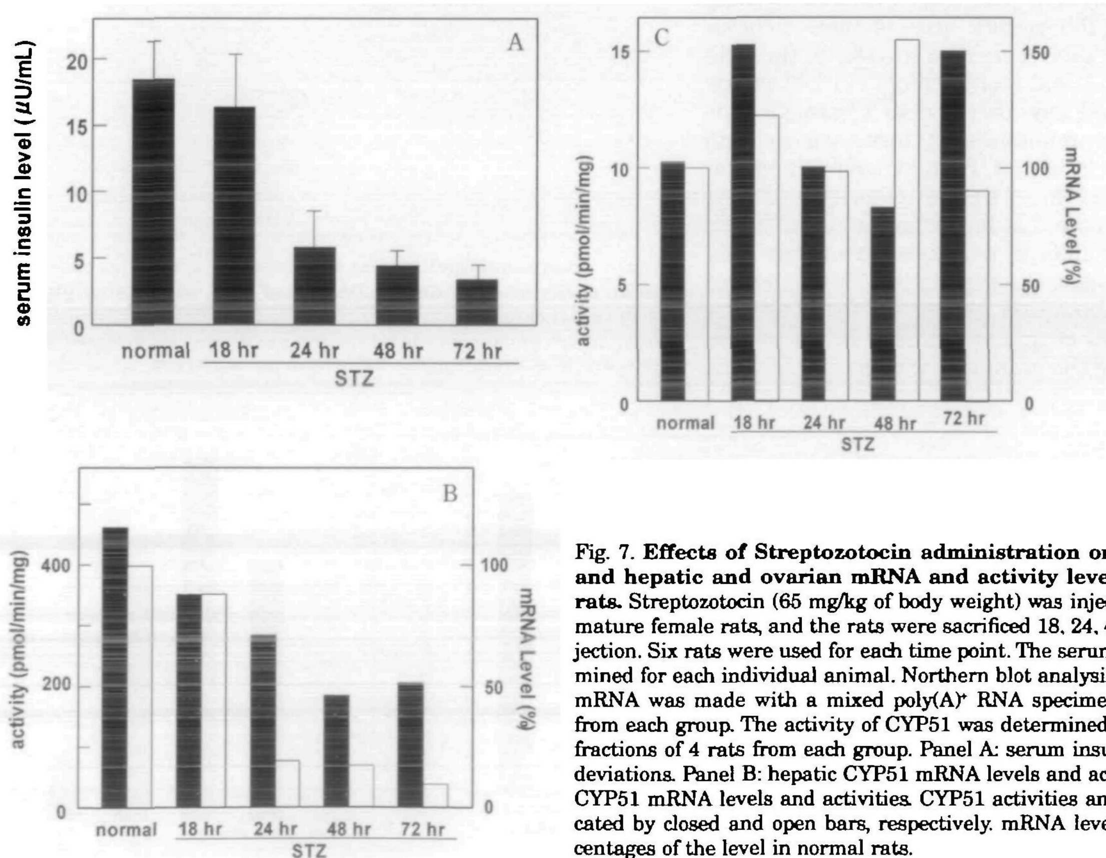

Fig. 7. **Effects of Streptozotocin administration on serum insulin levels and hepatic and ovarian mRNA and activity levels of CYP51 in female rats.** Streptozotocin (65 mg/kg of body weight) was injected subcutaneously into mature female rats, and the rats were sacrificed 18, 24, 48, and 72 h after the injection. Six rats were used for each time point. The serum insulin level was determined for each individual animal. Northern blot analysis for determining CYP51 mRNA was made with a mixed poly(A)$^+$ RNA specimen prepared using 2 rats from each group. The activity of CYP51 was determined with the combined S-10 fractions of 4 rats from each group. Panel A: serum insulin levels with standard deviations. Panel B: hepatic CYP51 mRNA levels and activities. Panel C: ovarian CYP51 mRNA levels and activities. CYP51 activities and mRNA levels are indicated by closed and open bars, respectively. mRNA levels are expressed as percentages of the level in normal rats.

DISCUSSION

CYP51 is expressed ubiquitously in most mammalian organs (*5, 6*). This enzyme has been recognized as an essential enzyme catalyzing the 14-demethylation of sterol precursors, such as lanosterol (*1*). However, Byskov *et al.* (*11*) reported that the 14-demethylated derivatives of lanosterol have the function of MAS. CYP51 is the only known enzyme that catalyzes the 14-demethylation of lanosterol (*1*) and ovarian CYP51 in immature rats induced with PMSG (*12*). These facts suggest that ovarian CYP51 may have an ovary-specific function, and that ovarian CYP51 may be regulated by pituitary gonadotropins.

Ovarian CYP51 is expressed dominantly in follicles and corpus lutea (Fig. 2). Hypophysectomy suppresses the expression of ovarian CYP51, and PMSG induces ovarian CYP51 together with ovarian growth (Figs. 3 and 4). The induction of ovarian CYP51 activity by PMSG is due to the synergism of a marked growth of follicles and the elevation of CYP51 levels in each follicle (Fig. 4). After the 2nd day of PMSG treatment, luteinization and the expression of CYP51 in corpus lutea together with further increases in ovarian CYP51 activity were observed (Figs. 3 and 4). The latter event might be due to the LH action of PMSG, which was confirmed by hCG treatment of PMSG-pretreated hypophysectomized rats (Fig. 5). Taken together, these observations lead to the conclusion that the expression of ovarian CYP51 is dependent on pituitary gonadotropins. CYP51 expression in follicles may be dependent on FSH, and that in corpus lutea may occur on luteinization triggered by LH. Rats have an incomplete and short (4 days) estrus cycle, and their ovaries usually contain both growing follicles and corpus lutea. Therefore, the observed ovarian CYP51 activity in a mature rat is the sum of that in the follicules and lutea. Immunohistochemical analysis revealed that CYP51 is not expressed in follicular oocytes. It has been reported (*10*) that CYP51 in male germ cells is expressed only in postmeiotic spermatids, and meiosis in oocytes is completed after ovulation. These facts suggest that CYP51 in germ cells is expressed after the completion of meiosis.

Sterol analysis of mature rat ovaries revealed that about 10 pmol/mg of wet tissue of FF-MAS is accumulated in mature rat ovaries (Table I). This amount suggests that the apparent FF-MAS concentration in ovaries is not less than 10 μM, which is enough to induce the resumption of meiosis (*11*). Sterol analysis also showed that the content of FF-MAS is considerably higher than those of lanosterol and T-MAS (Table I). FF-MAS is formed from lanosterol by CYP51 and T-MAS is the reduced metabolite of FF-MAS by sterol 14-reductase (*13*). Inhibition of the latter enzyme in cultured mouse follicles causes an accumulation of FF-MAS (*14*). Therefore, the specific accumulation of FF-MAS in rat ovaries must be due to a relatively higher activity of CYP51 than the reductase. Ovarian CYP51 activity shows large variations (Fig. 1), and this may be related to the larger variations in FF-MAS and T-MAS contents than in the lanosterol content (Table I), suggesting a relationship between the ovarian CYP51 level and ovarian MAS accumulation. Recently, Leonardsen *et al.* (*14*) inferred that the direct transference of FF-MAS produced in cumulus cells to oocytes is necessary for stimulating meiosis based on the observation that intact oocyte-cumulus cell connections are essential for stimulating meiosis by the accumulation of FF-MAS resulting from the inhibition of metabolism. These facts seem to suggest that a local elevation of FF-MAS concentration in cumulus cells enclosing an oocyte induces the resumption of meiosis. As shown in Figs. 2A and 4C, the immunohistochemically detected CYP51 expression level is slightly but clearly higher in cumulus cells and medial granulosa cells close to the antrum than in peripheral granulosa cells. The elevated CYP51 level in these cells supports the local elevation of FF-MAS concentration in cumulus cells and/or follicular fluid necessary for the resumption of meiosis. Ovarian CYP51 activity tends to increase in diestrus or proestrus, the stage at which the final maturation of preovulatory follicles occurs (Fig. 1). This observation may also be related to the elevation of FF-MAS production leading to the resumption of meiosis (*14*).

CYP51 is highly expressed in corpus lutea. Since one of the important functions of corpus lutea is progesterone production, a possible reason of the high expression of CYP51 in corpus lutea may be the supply of cholesterol necessary for progesterone synthesis. However, it has been reported that the induction of luteal steroid hormone synthesis triggered by gonadotropins is dependent on the elevation of steroidogenic acute regulatory protein (StAR) and low-density lipoprotein receptor (LDL-R) levels (*20*). This indicates that the cholesterol necessary for the production of steroid hormones upon luteinization is dominantly supplied from LDL. Consequently, cholesterol supply by *in situ* production may not play a dominant role in luteal steroid hormone synthesis. On the other hand, FF-MAS has been reported to be a potent ligand of the nuclear receptor LXRα which is a regulatory protein for several sterol metabolizing enzyme genes (*21*); the presence of the mRNAs for LXRα and retinoid-X receptor (RXR), the heterodimeric partner of LXRα (*22*), in rat ovaries has been detected by RT-PCR and northern blotting (Noshiro *et al.*, unpublished). These facts seem to suggest the possibility that the high expression of CYP51 and the resulting accumulation of FF-MAS in corpus lutea may play a certain regulatory role coupled with LXRα. Thus, further studies on luteal CYP51 are necessary to understand the regulation of luteal sterol synthesis and the possible role of FF-MAS as a regulatory molecule in corpus lutea.

The accumulation of FF-MAS is an important function of ovarian CYP51. On the other hand, considerable amounts of T-MAS and little FF-MAS accumulate in rat testes (Yamashita *et al.*, unpublished), a result that agrees with the findings of Byskov *et al.* (*11*) that FF-MAS is isolated from follicular fluid and T-MAS is obtained from testes. These facts suggest that FF-MAS and T-MAS participate in organ-specific functions in ovary and testis, respectively. Accordingly, the organ-specific regulation of *CYP51* expression may be important not only for regulating sterol production in each organ but also for accumulating metabolites as organ-specific regulatory substances. The expression of hepatic *CYP51* is supported by serum insulin (*7*), but the expression of ovarian *CYP51* is not affected by serum insulin (Fig. 7). The expression of ovarian *CYP51* is dependent on gonadotropins, but PMSG has no effect on hepatic *CYP51* expression in hypophysectomized rats (Yamashita *et al.*, unpublished). The expression of *CYP51* in male germ cells is regulated by CREMτ (*9*), and the

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expression of testicular *CYP51* is independent of serum insulin (*7*). The expression of *CYP51* in vascular endothelial cells is repressed by LDL through an SREBP-2-dependent mechanism (*23*). These facts indicate that the regulation of *CYP51* differs among organs. *CYP51* is a housekeeping gene with no TATA-box and its transcription is started at multiple sites that may be linked to several SP-1 binding elements (GC-box) scattering in the promoter region (*6*). However, a few consensus sequences of regulatory elements, such as CRE, SRE-1, and GATA-1, have been identified in the promoter region of *CYP51*. Since *CYP51* is a single copy gene (*5, 6*), the organ-specific regulation of *CYP51* expression may be achieved by different usages of these elements. The identification of regulatory elements and proteins regulating *CYP51* expression in each organ is an interesting subject for future studies.

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