Genetic Variation in Plasma Androgens and Ovarian Aromatase Activity During Mouse Pregnancy (43856)

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Abstract. **Genetic variation in fetal survival, maternal plasma androgen levels, and ovarian aromatase activity was examined mid (Day 9) and late gestation (Day 16) in strains of mice that differ in reproductive performance (A/J, C57BL/6J, C8/Jls, C17/Jls, and S15/Jls). At both gestational stages, females selected for large litter size (S15/Jls) carried more fetuses than any of the other strains examined. Particularly at midpregnancy, S15/Jls females also maintained higher plasma levels of androstenedione and testosterone relative to both control strains, C8/Jls and C17/Jls. Consistent with previously reported changes in peripheral estrogen levels during mouse pregnancy, aromatase activity was higher on Day 16 than on Day 9. This study demonstrates genetic variation in fetal survival that is correlated with increased maternal androgen levels. A stage-specific gestational increase in aromatase activity occurs in several strains of mice and is associated with elevated plasma estrogen during the second half of pregnancy.** [P.S.E.B.M. 1995, Vol 208]

The endocrine transition from maternal to fetal-placental control of pregnancy is associated with embryo loss in several mammalian species including humans (1, 2). In the laboratory mouse, a marked increase in circulating androgen occurs precisely when maternal regulation of luteal function is assumed by the conceptuses at midgestation (3, 4). The midgestational androgen surge occurs at a discrete stage of fetal-placental development (5) and is characterized by marked genetic variation associated with fetal survival (6). During the last week of mouse pregnancy, maternal androgen levels increase a second time (6).

A few days after the midgestational surge of androgen on Days 9–10, maternal levels of estradiol begin to increase and eventually parallel the late gestational rise in androgen (6). The pattern of progesterone secretion is similar, increasing from midpregnancy until shortly before parturition. Genetic differences in maternal concentrations and secretory profiles of testosterone, estradiol, and progesterone become dramatic at midgestation and are striking during the second half of pregnancy (6). Specifically, strains selected for improved reproductive performance have sharply defined, pronounced surges of testosterone and higher maternal concentrations of estradiol and progesterone at mid and late gestation. This suggests that the fetal-placental-ovarian unit changes more readily following genetic selection than does pituitary control over ovarian steroid biosynthesis. Among many possibilities, one hypothesis is that differences in maternal estradiol production reflect changes in ovarian aromatase activity which have occurred as a correlated response to selection for another reproductive trait like large litter size.

The aromatase enzyme complex, commonly referred to simply as *aromatase,* consists of an aromatase cytochrome P450 and a flavoprotein, NADPH-cytochrome P450 reductase. Aromatase cytochrome P450 (P450arom), currently referred to as CYP19 (7), converts Δ^4-androstenedione to estrone, testosterone to estradiol, and 16-hydroxyandrostenedione to es-

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triol. The aromatase enzyme complex has been localized to murine granulosa cells (8, 9) and luteal cells in the pregnant rat (10). The present study investigated fetal survival and ovarian aromatase activity at mid (Day 9) and late gestation (Day 16) in strains of mice that vary in reproductive characteristics.

Materials and Methods

Animals. Mice were obtained from two sources: strains A/J and C57BL/6J were purchased from the Jackson Laboratory, Bar Harbor, Maine. The other strains of mice were developed at the University of California, Davis, the University of Michigan, and the University of Wisconsin, from a common base population consisting of a cross of four inbred strains (C57BL/6, AKR/J, C3H/J, and DBA/2). A randomly bred control line (C) was maintained by random selection (11). Line S1 was successfully selected for large litter size in a closed, noninbred population for over 60 generations (15). The present study utilized control inbred strains C8/Jls and C17/Jls which are randomly bred strains derived by over 40 generations of inbreeding from line C. A large litter size strain, S15/Jls, inbred from line S1 for over 50 generations with continued selection for large litter size, was also used in the present study. Although the strains of mice used in the present study are not identical to those in which gestational hormone levels were examined (6), strains C8/Jls and C17/Jls represent the range of variation of the control line (C). S15/Jls closely resembles S1, since inbreeding from line S1 to develop strain S15 was started after 34 generations of selection for large litter size.

Mice were housed in the same animal room maintained at 21°–23°C and illuminated for 14 hr/day (lights on from 0500–1900 hr). Purina Rodent Chow and water were provided *ad libitum*. At 60–70 days of age, nulliparous females were placed in cages with a male of their own strain and checked daily for the presence of a copulatory plug (Day 0 of pregnancy). After a vaginal plug was detected, the bred female was housed individually or with other females of the same strain that had mated during the same period. On Days 9 and 16 of pregnancy, females of each strain were decapitated between 1000 and 1200 hr, and the trunk blood was collected over heparinized funnels. After refrigerated centrifugation (20 min at 1000g), the plasma was recovered and stored at −20°C until assayed for testosterone and androstenedione. The small sample volume obtained from individual mice precluded the assay of additional hormones. Immediately after decapitation, the ovaries were dissected from adhering fat pad, rapidly frozen in a solid CO_2/acetone bath and stored at −70°C. At time of dissection, the reproductive tract was removed and the number of viable fetuses from each uterine horn was recorded.

Quantification of Steroid Hormones. Plasma androstenedione and testosterone were measured using commercially available, solid-phase radioimmunoassay kits specific for each androgen. Dilution-response curves for a mouse plasma pool were generated to verify the use of these assays for mouse androgens. Comparison of the slopes of logit transformation with linear regression documented that the pooled mouse plasma, androstenedione, and testosterone standard curves were parallel. The quantification of androstenedione was performed using a highly specific rabbit anti-androstenedione immunoglobulin (Catalog no. DSL 3800; Diagnostic Systems Laboratories, Inc., Webster, TX). The antiserum cross-reacts less than 1% with other androgens, 0.08% with estrone, 1.19% with 11-deoxycortisol, and less than 1% with other estrogens, progestins, or corticosteroids. The sensitivity of the assay is 0.1 ng/ml at the 95% confidence limit, and the intra- and interassay coefficients of variation are 4.1% and 7.60%, respectively. Testosterone was measured using a highly specific antiserum (Catalog no. TKTT1; Diagnostic Products Corp., Los Angeles, CA) which cross-reacts 3.3% with dihydrotestosterone, 0.5% with androstenedione, less than 1% with other androgens, 0.2% with estradiol, 0.002% with corticosterone, and not at all with progesterone. The sensitivity of the assay is 0.04 ng/ml at the 95% confidence limit, and the intra- and interassay coefficients of variation are 5% and 9.2%, respectively.

Estradiol was quantified by a specific enzyme-linked immunosorbent assay (ELISA) according to modifications of the procedure of Spearow and Trost (12). Briefly, flat-bottomed, 96-well polystyrene plates (Nunc Immuno Plate I 439454) were coated with Protein A (1 μg/ml), to which were added anti-estrogen sera 482-8A (1:45,000) obtained as a gift from Dr. O. D. Sherwood, University of Illinois, Urbana, IL. Plates were washed four times with wash buffer [0.02 *M* $NaPO_4$, 0.12 *M* NaCl, 0.025% polyoxyethylene sorbitan monolaurate (Tween 20), pH 7.2]. Estrogen standards and samples were added in triplicate to the microwell plates in assay buffer (0.02 *M* MOPS, 0.12 *M* NaCl, 0.1% gelatin, 0.05% Tween 20, 0.01 *M* EDTA, 0.005% chlorhexidine diacetate, 0.002% phenol red, pH 7.2). Estrogen-3-peroxidase was then added in assay buffer and the plates were incubated for 2 hr at 20°C. Plates were subsequently rinsed with wash buffer to remove unbound estrogen, after which was added 100 μl of peroxidase substrate buffer [0.5 ml of 3,3′,5,5′-tetramethylbenzidine (TMB)] stock solution [20 mg TMB/ml dimethyl sulfoxide (DMSO)], 0.16 ml 0.5 M H_2O_2, 49.34 ml 0.05 *M* Na acetate, pH 4.8] prepared immediately before use. The reaction was terminated with 50 μl of 0.5 *M* sulfuric acid, and the color produced was measured (optical density 450–600 nm)

with a Molecular Devices microplate autoreader. Estrogen concentrations were estimated with a four parameter logistic curve using the Soft Max microplate data analysis computer program.

Aromatase Activity. Aromatase activity was measured by quantification of the estradiol produced by ovarian preparations incubated with a NADPH regenerating system and the androgen substrate, testosterone. Ovaries from each mouse were homogenized in steroidogenic buffer [20 m*M* N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid (TES), 10 m*M* EDTA, 150 m*M* KCl, and 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), pH 7.3] and then centrifuged at 2000g for 5 min. In a preliminary experiment, aliquots of the 2000g supernatant were centrifuged at 40,000g for 30 min, the pellets were resuspended in steroidogenic buffer and then assayed for aromatase activity. The small size of individual mouse ovarian samples and apparent enzyme losses during the preparation of the 40,000g pellet resulted in a consistent reduction in aromatase activity in the resuspended microsomal fraction. Therefore, an aliquot of the 2000g supernatant was assayed directly for aromatase activity. Ovarian preparations from each mouse were incubated in duplicate with 5 μM testosterone, as substrate, for 10 min in a 37°C water bath. A NADPH regenerating mix (0.5 m*M* NADPH, 0.5 m*M* NADH, 6 m*M* glucose-6-phosphate, and 1 unit/ml glucose-6-phosphate dehydrogenase) was provided in the incubation to ensure availability of NADPH. The steroidogenic reaction was terminated by placing the incubation in a boiling water bath for 5 min. The tubes were then capped and stored at −20°C until estradiol was quantified by immunoassay (ELISA). Aromatase activity was calculated by subtracting the estradiol concentration in the 0-time incubation (which detects endogenous estrogen) from the 10-min incubation.

Data Analysis. Data were subjected to analyses of variance, followed by Tukey-Kramer comparisons to determine differences between strains on Days 9 and 16 of gestation. Prior to these analyses, a level 1 Winsorization (13) was performed to adjust for the extreme variation in aromatase activity within and between strains. The regression of androstenedione and testosterone on fetal number was also examined on Days 9 and 16 of gestation. Only differences between means at $P \leq 0.05$ were considered significant.

Results

Fetal number *in utero* is shown in Figure 1. On both Days 9 and 16 of gestation, S15/Jls females supported a greater number of viable fetuses (13.4 ± 0.45; 12.8 ± 0.83, respectively) than females of any other strain (Ps < 0.01). Maternal plasma concentrations of androstenedione and testosterone are shown in Figures 2 and 3. In general, androgen levels were higher

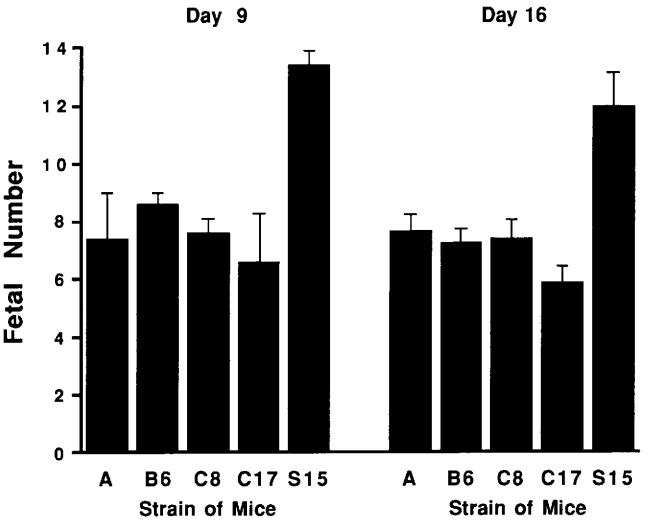

Figure 1. Number of viable fetuses on Days 9 and 16 of gestation in females of strains A/J (A); C57BL/6 (B6); C8/Jls (C8); C17/Jls (C17); and S15/Jls (S15). The columns and vertical bars represent the mean and SE, respectively, for each group. The number of females of each strain sampled on Day 9 or 16 of pregnancy is as follows: Day 9—A = 5; B6 = 5; C8 = 8, C17 = 5; S15 = 9. Day 16—A = 6; B6 = 7; C8 = 7, C17 = 8; S15 = 7.

on Day 9 than on Day 16 of gestation (Ps < 0.05). Marked strain differences in the levels of androstenedione were present. C57BL/6J and S15/Jls mice secreted significantly more androstenedione on Day 9 (3.1 ± 0.60; 2.6 ± 0.33 ng/ml, respectively) than did females of strains C8/Jls (0.84 ± 0.07 ng/ml) and C17/Jls (0.85 ± 0.28 ng/ml) (Ps < 0.05) (Fig. 2). Androstenedione levels were also higher on Day 16 of pregnancy in S15/Jls females (1.4 ± 0.15 ng/ml) than in other strains with the exception of C8/Jls (0.98 ± 0.09 ng/ml). Although C57BL/6J mice had relatively high

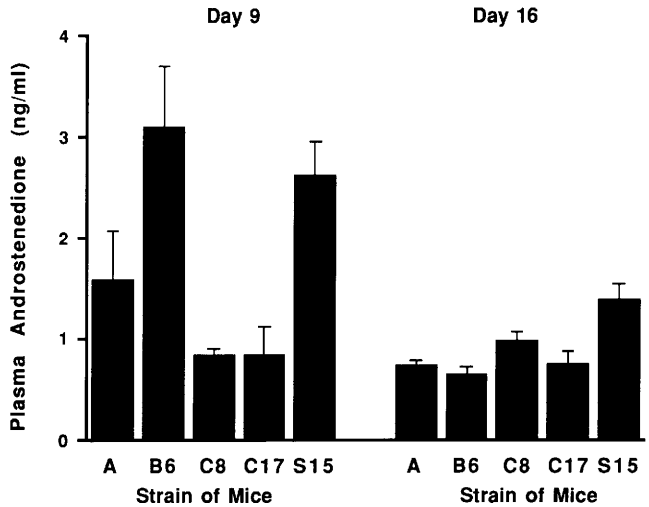

Figure 2. Maternal plasma androstenedione concentrations on Days 9 and 16 of gestation in strains A/J (A); C57BL/6 (B6); C8/Jls (C8); C17/Jls (C17); and S15/Jls (S15). The columns and vertical bars represent the mean and SE, respectively, for each group. The number of females of each strain sampled on Day 9 or 16 of pregnancy is given in the legend for Figure 1.

androstenedione concentrations on Day 9 (3.1 ± 0.60 ng/ml), a dramatic reduction in androstenedione secretion occurred by Day 16 in this strain (0.64 ± 0.07 ng/ml) ($P < 0.05$). A similar decrease in androstenedione concentrations occurred by Day 16 in S15/Jls females (1.4 ± 0.15 ng/ml) ($P < 0.05$).

Genetic variation in maternal levels of testosterone during mid and late gestation was also observed (Fig. 3). Consistent with strain differences in androstenedione levels, C57BL/6J and S15/Jls mice secreted more testosterone on Day 9 (0.61 ± 0.09; 0.59 ± 0.06 ng/ml, respectively) than did females of strains C8/Jls (0.23 ± 0.02 ng/ml) or C17/Jls (0.16 ± 0.04 ng/ml) (Ps < 0.05). As was the case for androstenedione, testosterone was higher in the maternal circulation at midgestation (Day 9) than on Day 16, by which time strain differences in testosterone concentrations had disappeared.

Ovarian aromatase activity on Days 9 and 16 of pregnancy is shown in Figure 4. Across strains, ovarian aromatase activity was higher on Day 16 than on Day 9 of pregnancy ($P < 0.01$). Within strains, ovarian aromatase activity was significantly higher on Day 16 than on Day 9 in C57BL/6J, C8/Jls and C17/Jls mice (Ps < 0.05). A similar trend was seen within strains A/J and S15/Jls, but ovarian aromatase activity was not significantly greater on Day 16 than on Day 9 within either strain.

On Day 9, ovaries harvested from S15/Jls mice aromatized more androgen to estradiol than ovaries recovered from any other strain ($P < 0.05$). Strain differences in aromatase activity were also present on Day 16. C17/Jls ovaries collected at this stage of gestation converted more androgen to estradiol than did

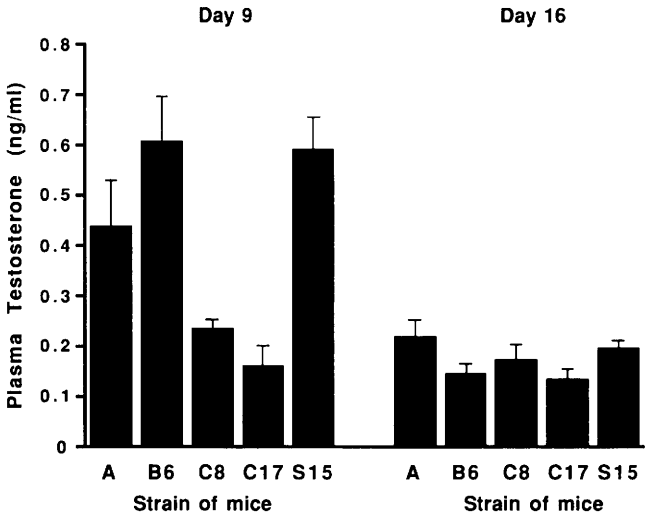

Figure 3. Maternal plasma testosterone concentrations on Days 9 and 16 of gestation in strains A/J (A); C57BL/6 (B6); C8/Jls (C8); C17/Jls (C17); and S15/Jls (S15). The columns and vertical bars represent the mean and SE, respectively, for each group. The number of females of each strain sampled on Day 9 or 16 of pregnancy is provided in the legend for Figure 1.

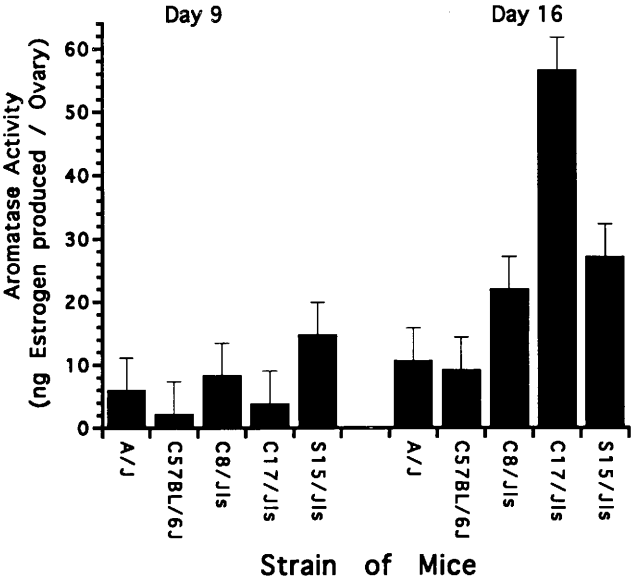

Figure 4. Ovarian aromatase activity per ovary per 10-min incubation on Days 9 and 16 of gestation in strains A/J (A); C57BL/6 (B6); C8/Jls (C8); C17/Jls (C17); and S15/Jls (S15). The columns and vertical bars represent the mean and SE, respectively, for each group. The number of females of each strain from which ovarian homogenates were prepared on Day 9 or Day 16 of pregnancy is as follows: Day 9, A = 5; B6 = 6; C8 = 8; C17 = 5; S15 = 9. Day 16, A = 4; B6 = 5; C8 = 6; C17 = 4; S15 = 4.

ovaries obtained from all other strains of mice ($P < 0.05$). Thus, the relative difference in aromatase activity in strain C17/Jls reversed from Day 9 where it was among the lowest, such that by Day 16 it was the highest.

Discussion

Embryo mortality is a major cause of reproductive inefficiency and failure. During pregnancy, changes in hormone production in mice are correlated with changes in embryo survival and the number of viable offspring produced. For example, elevated maternal concentrations of steroid hormones, in particular androgens and estrogens, are found in strains with high reproductive performance, while low levels of steroids are characteristic of strains with poor reproductive success (6). Interestingly, embryo survival can be increased in mice with small litters if therapy with exogenous progesterone is initiated during the first half of pregnancy when luteal production of progesterone is supported by the maternal pituitary (14). Supplementation with exogenous progesterone after midgestation does not substantially increase embryo survival because the majority of fetal wastage has occurred by this time (14, 15).

The transition from maternal to fetal-placental regulation of luteal function occurs at midgestation on approximately Days 9–10 in the mouse (4) and Days 10–12 in the rat (16). During this transitional period, testosterone and androstenedione are produced by the ovary (17, 18) and the placenta (19, 20). The androgen provides substrate for the synthesis of ovarian estro-

gen which acts to sustain luteal progesterone production by an intracrine mechanism (21, 22). This fetal-placental-ovarian unit is operative by midgestation, the time when hypophysectomy can be accomplished without the interruption of gestation (4, 16). Prior to this time, the ovary is the principal source of androgen substrate for aromatization to estrogen (18), luteinizing hormone providing the stimulus for luteal androgen production (23). Prolactin and prolactin-like hormones maintain luteal cell content of both luteinizing hormone and estradiol receptors, thereby controlling both the formation and action of estradiol in the luteal cell (22). Aromatase cytochrome P450 (P450arom) increases in rat luteal cells from midpregnancy until shortly before parturition (Day 10–19 of pregnancy) (10). Although our measurement of ovarian aromatase activity was restricted to Days 9 and 16 of pregnancy, in all strains examined aromatase activity was highest during late gestation. This suggests that P450arom may also increase steadily in mouse luteal cells after midgestation, as it does in the pregnant rat (10). As gestation progresses, the ovary maintains its aromatase activity (18) but loses its ability to convert progesterone to androstenedione, and the placenta eventually becomes the principal source of androgen substrate for aromatization to estradiol.

The present study addressed the possibility that genetic variation in the production of androgen and estrogen during mid and late pregnancy is associated with strain differences in fetal number and ovarian aromatase activity. At Day 9, the regression of both androstenedione and testosterone on fetal number was highly significant ($P < 0.01$). At Day 16, the regression of androstenedione on fetal number was also significant ($P < 0.05$). Although the number of corpora lutea (CL) was not determined in the present study, previous characterizations of strain differences in the number of CL largely parallel strain differences in the number of fetuses observed on Days 9 and 16 (24). Thus, genetic differences in plasma androgens were largely associated with strain differences in both the number of CL and fetuses. However, during the first half of pregnancy, embryo survival in C57BL/6J was not influenced by the production of androgen substrate. On Day 9, maternal concentrations of androstenedione and testosterone in C57BL/6J resembled those found in S15/Jls, the line that supported a significantly larger number of fetuses than any other strain examined.

Fetal survival at midgestation does not appear limited by genetic differences in ovarian aromatase activity per se. Aromatase activity reversed in strain C17/Jls from among the lowest at Day 9 to the highest at Day 16. This supports the idea that different genetic controls of aromatase activity are operative at Days 9 and 16 in the strains of mice we examined. It has already been demonstrated that mice have different RFLP alleles at the Cyp-19 locus which codes for the P450aromatase structural gene (25, 26). Furthermore, the expression of P450arom is modulated by a variety of factors including cAMP, phorbol esters, glucocorticoids, and several growth factors (27–32), the interaction of which could change following genetic selection. In related studies examining genetic differences in hormone-induced follicular steroidogenesis between A/J and B6 mice, a B6 gene controlling major differences in aromatase activity mapped to a trans-acting quantitative trait locus on Chromosome 4 but not to *Cyp*-19 on Chromosome 9 (33). Apparently, the regulation of ovarian aromatase activity is complex involving several genes and physiological factors that act and interact to control ovarian estrogen production. It is also clear that steroidogenic capacity is only one of several variables that play important roles in the determination of embryo survival.

In summary, the present study confirms and extends genetic variation in fetal number, maternal androgen concentrations, and ovarian aromatase activity during mouse gestation. Genetic variation in maternal androgen levels may account, at least in part, for gestational differences in maternal steroid levels and fetal survival. The differences in circulating androgen and ovarian aromatase activity are most pronounced at midgestation, the period when fetal-placental control of luteal progesterone production is initiated and fetal wastage is nearing completion.

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