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Genetics

Genetic control of renin activity in the submaxillary gland of the mouse

(androgen action/angiotensin/regulatory genes)

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ABSTRACT **Administration of androgen to female mice is known to increase the level of several proteins in the submaxillary gland, including nerve growth factor, epidermal growth factor, esteroproteolytic activity, and renin. In the present study, renin activity has been assessed in extracts of submaxillary gland of female mice from two inbred strains (SWR/J and C57BL/10J), from F_1 and F_2 hybrids, and from backcrosses between F_1 and parental strains. In both uninduced and induced mice, renin activity of submaxillary gland was more than 100-fold greater in SWR/J than in C57BL/10J mice as measured by either an enzymatic assay or an immunodiffusion method. This difference was not due to differences in plasma testosterone levels between the strains, and the enzymes from the two strains had similar pH optima, substrate specificities, heat stabilities, and apparent Michaelis constants. In the submaxillary gland the difference was relatively specific for renin because increases in esteroproteolytic activity, nerve growth factor, and epidermal growth factor after androgen treatment appeared to be similar in both strains. Studies with the various hybrids indicate that the difference in renin activity between the two strains is apparently due to a single regulatory gene.**

Swank, Paigen, and Ganschow (1) have recently reported that the induction of β-glucuronidase in mouse kidney by androgens is mediated by a regulatory gene that is believed to be located near the structural gene for the enzyme. This type of control could be a unique property of the β-glucuronidase system or, alternatively, individual regulatory genes might be involved in the mediation of each action of steroid hormones within target tissues.

In the mouse, several other enzymes and growth factors are regulated by androgens, including renin (2, 3), esteroproteolytic enzymes (4, 5), nerve growth factor (6), and epidermal growth factor (7) in the submaxillary gland and alcohol dehydrogenase (8) in the kidney. The regulation of renin-like enzymes of the submaxillary gland has been studied in considerable detail. In this gland, renin appears to exist as several isozymes with molecular weights between 36,000 and 43,000 (9). The enzyme acts on angiotensinogen to release angiotensin I (Ang I), the precursor of angiotensin II which is a pressor agent with potent hypertensive properties (10–13). Renin is present in the granulated ducts of the gland (14, 15). In the intact tissue, the enzyme is located predominantly in the granules and is released into the soluble fraction upon homogenization (16). There is a profound dimorphism in this activity, the submaxillary glands of males having a much higher content than those of females. This difference is the result of androgen action because the administration of physiologically active androgen increases the activity in glands from castrated males and from females to levels seen in mature males (2, 3). This enhancement of activity by androgens is clearly due to an increase in the synthesis of renin (17). Furthermore, Bing and his associates (18) have demonstrated with bioassay techniques that the activity in the submaxillary gland is under genetic control since mice from some inbred strains have high activity and others have low activity with intermediate levels in the F_1 hybrids.

In studying the genetic control of renin induction by androgens, we found that not only do certain inbred strains of mice have different levels of renin activity in the submaxillary gland but that they also respond differently to androgen. Our findings indicate that differences in renin activity between these strains is due to alterations of a single gene that apparently involves regulation of the enzyme. The gene is apparently specific in that similar differences were not observed in the response of esteroproteolytic activity, nerve growth factor, or epidermal growth factor in these strains.

MATERIALS AND METHODS

Treatment of Animals. The original stocks of inbred strains of mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The *Tfm* animals were bred from heterozygous female carriers that were the gift of Dr. S. Ohno (19). C57BL/10J and SWR/J mice were mated to produce F_1 hybrid offspring. Matings between C57BL/10J and F_1 hybrids and between SWR/J and F_1 hybrids produced backcross offspring. Matings between F_1 mice produced F_2 offspring. Female mice, 6 weeks ± 3 days old, were injected subcutaneously with 2 mg of dihydrotestosterone in 0.2 ml of sesame oil on days 0, 2, 4, and 6 (induced). Uninduced animals received equal volumes of sesame oil (17). Animals used to determine the time course of response were injected on alternate days for longer periods as indicated in Fig. 1. Eight-week-old male mice were studied without prior treatment.

Homogenization. Animals were killed on day 7 by severing the spinal cord. The submaxillary (submandibular) glands were cleaned, separated from the adherent sublingual glands, weighed, diced with a razor blade, added to 15 volumes of 20 mM Tris·HCl, pH 7.4, containing 1.5 mM EDTA, and homogenized in a micro-Dounce homogenizer by using 10 strokes each of a loose pestle (0.15-mm clearance), a medium pestle (0.08-mm clearance), and a tight pestle (0.05-mm clearance). All solutions were ice-cold. Homogenates were centrifuged at $104,000 \times g$ (40,000 rpm) for 60 min, and the supernatants were stored at −20°. Average weights of submaxillary glands were 52 and 85 mg (C57BL/10J) and 92 and 127 mg (SWR/J) in the uninduced and induced animals, respectively.

Assays. In the routine assay of renin activity, the final incubation mixture (total volume, 125 μl) contained supernatant (0.02–80 μg protein), 1 mM *o*-phenanthroline, 1 mM diisopropylfluorophosphate, 13 mM 2,3-dimercaptopropanol, 50 mM Tris·HCl, and either 1.8 mg of partially purified hog angiotensinogen containing the equivalent of 0.8 nmol of Ang I (final pH, 7.5) or 90 μl of nephrectomized rat serum containing the equivalent of 0.2 nmol of Ang I (final pH, 8.4). The

Abbreviation: Ang I, angiotensin I.

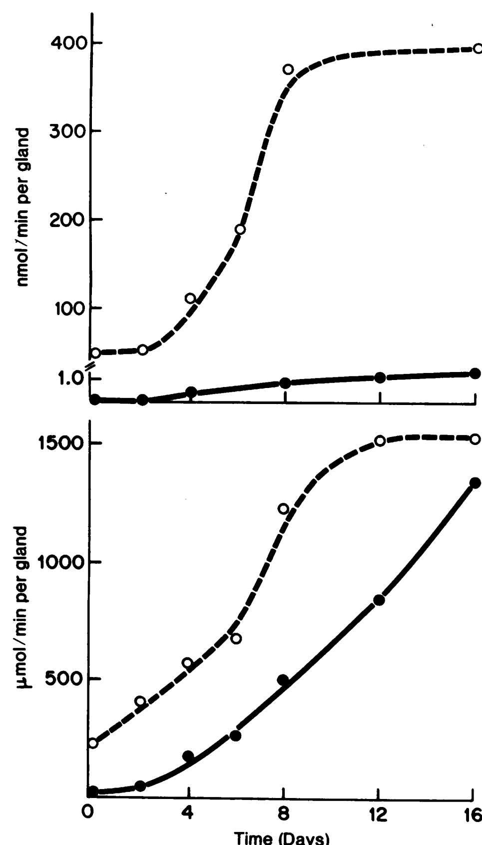

FIG. 1. Time course of induction of renin (*upper*) and esteroproteolytic (*lower*) activities in the submandibular gland of 6-week-old female SWR/J (O) and C57BL/10J (●) mice given 0.2 ml of triolein containing 0.2 mg of dihydrotestosterone by subcutaneous injection every other day for varying times. The animals were all killed on the same day, and the submandibular glands were removed and processed as described in the *text*. The average values from two animals are represented.

supernatants had been preincubated at a protein concentration of 0.6–1 mg/ml for 15 min at 37° in the presence of the *o*-phenanthroline/diisopropylfluorophosphate/mercaptopropanol/Tris mixture to inhibit Ang I-converting enzyme and angiotensinases. The reaction was initiated by adding aliquots of preincubated supernatant to the substrate. After incubation of the final mixture at 37° for 10 min the reaction was stopped by freezing at −50°.

In the study of the time course of induction and assays of kidney renin the concentration of hog renin substrate was suboptimal (i.e., a total of 0.2 nmol of Ang I available per incubation tube), and the enzyme reaction was done in 50 mM sodium phosphate, pH 6.8, instead of Tris. The reaction velocity was not maximal under these conditions, but the generation of Ang I was proportional to the amount of enzyme added. In the routine assay using hog angiotensinogen, the substrate concentration was about 3 times the apparent Michaelis constant, K_m, and less than 5% of the substrate was consumed during the incubation. A standard pool of submaxillary gland supernatant was included in each assay series, and results were corrected for any variation in this value. The mean (±SD) value for the reference supernatant in eight assays was 13.8 ± 2.8 nmol of Ang I generated per min per mg of protein (range, 9.9–17.0). For determination of the effect of pH on renin activity, the buffer was either 50 mM sodium phosphate (pH 6–6.8) or 50 mM Tris·HCl (pH 7.1–8.4) measured at 25°. In all instances the amount of Ang I generated was determined by radioimmunoassay (17) according to the procedure described in the Squibb Angiotensin I Immunotope Kit. Results are expressed as nmol of Ang I produced per min at 37°.

Table 1. Induction of renin activity in the submaxillary glands of female mice

Mice	Renin activity, nmol Ang I/min per mg protein	
	Untreated	Treated
C57BL/10J	0.0015 ± 0.0014	0.13 ± 0.10
SWR/J	9.7 ± 4.7	27 ± 5
F_1	1.6 ± 0.69	14 ± 2

The treated animals were given dihydrotestosterone in sesame oil for 7 days prior to death, and the uninduced animals were given sesame oil only for the same period. All studies were performed with hog renin as substrate. The numbers represent mean ±SD of values shown in Fig. 3.

Esteroproteolytic (tamase) activity was assayed by measuring the hydrolysis of α-*N*-tosylarginine methyl ester by the method of Levy, Fishman, and Schenkein (20). The unit of enzymatic activity is µmol of substrate hydrolyzed per min at 37°. Protein was measured by the method of Lowry *et al.* (21), and testosterone was measured by a radioimmunoassay procedure (22).

Reagents. α-*N*-Tosylarginine methyl ester was purchased from Sigma, and radioimmunoassay reagents were from Squibb. Hog renin substrate was prepared as described previously (23); 1 mg of substrate preparation produced a total of 0.45 nmol of Ang I when incubated with excess renin. Serum with a high angiotensinogen concentration (2.3 nmol Ang I equivalents per ml) was obtained from male Sprague-Dawley rats that had been nephrectomized 16–20 hr earlier (24); immediately prior to use, the pH was adjusted to 8.5 (13) and the serum was incubated at 37° for 15 min in the presence of 1 mM *o*-phenanthroline/1 mM diisopropylfluorophosphate/13 mM 2,3-dimercaptopropanol.

Antiserum to nerve growth factor from mouse submaxillary gland was obtained from Burroughs Wellcome Co. (Pa-07); antisera to renin and epidermal growth factor from mouse submaxillary gland were a gift from Drs. T. Inagami and S. Cohen, Vanderbilt University. Immunodiffusion plates (pattern C) were obtained from Hyland Laboratories.

RESULTS

In the course of a survey of the induction of renin and esteroproteolytic activities in the submaxillary glands of inbred strains of mice, a striking difference was observed in the effects of androgen on the two activities in females of the SWR/J and C57BL/10J strains (Fig. 1). Both enzymatic activities were higher in control SWR/J than in C57BL/10J females. After 8 days of dihydrotestosterone treatment, renin activity in SWR/J ("high-renin") mice rose from 49 nmol/min per gland to 375 nmol/min per gland and changed little thereafter, whereas the activity in C57BL/10J ("low-renin") mice was less than 2 nmol/min per gland after 16 days of treatment. Despite the fact that the activities in both treated and untreated C57BL/10J mice were low, androgen increased the activity by approximately two orders of magnitude either when expressed as activity per gland (Fig. 1) or per mg of protein (Table 1). No change was noted after androgen administration to the *Tfm* mice which are known to be androgen-resistant (25, 26) (0.043 and 0.050 nmol of Ang I/min per mg protein with and without

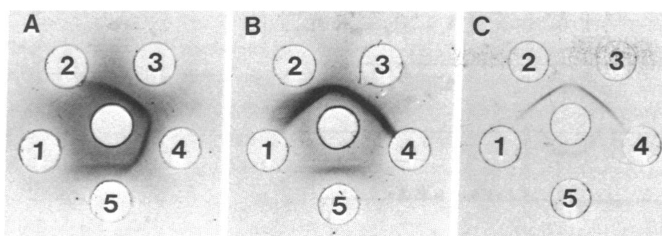

FIG. 2. Immunodiffusion of submaxillary gland supernatants from induced and uninduced C57BL/10J and SWR/J females with antisera to renin (*A*), epidermal growth factor (*B*), and nerve growth factor (*C*). The outer wells were filled with 10 μl of pooled supernatant from three to five animals; the center well contained 10 μl of saline with antiserum to either renin (0.5 mg of protein), epidermal growth factor (0.7 mg of protein), or nerve growth factor (0.25 mg of protein). The plates were allowed to sit at room temperature overnight, placed in isotonic saline for an additional day, stained for 0.5 hr with Amido-Black (0.05%) in methanol/acetic acid/H_2O, 5:4:1 (vol/vol), destained for about 16 hr in methanol/acetic acid/H_2O, 45:10:45 (vol/vol), and dried. *1*, uninduced C57BL/10J (3.8 mg of protein per ml); *2*, induced C57BL/10J (3.7 mg of protein per ml); *3*, induced SWR/J (4.9 mg of protein per ml); *4*, uninduced SWR/J (3.3 mg of protein per ml); *5*, induced SWR/J diluted 1:10 with saline.

androgen treatment). In contrast, after treatment with androgen, esteroproteolytic activity increased in low-renin mice in a fashion that was similar to the increase observed in high-renin animals (Fig. 1). The relatively low renin activity in C57BL/10J mice after treatment with dihydrotestosterone therefore is not due to a general inability of this strain to respond to androgen.

Similar differences in renin activity between the two strains were also observed in supernatants of submaxillary glands from male mice (0.9 and 835 nmol/min per gland for C57BL/10J and SWR/J, respectively). It is of interest, however, that no significant difference was seen in the renin activity of kidney supernatants from the two strains before or after androgen treatment. Testosterone levels in plasma of the uninduced female mice were similar (0.37 ng/ml in high-renin and 0.42 ng/ml in low-renin mice).

The differences in activities of submaxillary gland renin between the induced and uninduced animals of both strains were virtually the same when nephrectomized rat serum was used for the assay of pooled samples from three to five mice (0.006 and 0.37 and 8.4 and 38 nmol Ang I/min per mg of protein for untreated and treated low-renin and high-renin mice). That similar results were found with serum as substrate indicated that the induced activity is due to renin rather than pseudorenin (27). The pH optimum of renin activity was broad in submaxillary gland homogenates of both induced and uninduced low-renin and high-renin animals, ranging between pH 7.4 and 8.4 when hog renin substrate was used (data not shown). The apparent K_ms of the enzymes from induced and uninduced mice of both strains ranged between 1 and 2 μM Ang I equivalents with hog renin as substrate. When equal amounts of supernatant activity from the two strains were mixed prior to assay, an intermediate level of activity was observed, and when the two supernatants were heated for varying times at 52°, a similar decrease in activity was observed (results not shown).

When submaxillary gland supernatants were tested with antirenin antibody by the double diffusion technique of Ouchterlony (28), a clear-cut precipitin band formed with samples from induced and uninduced high-renin mice and with the extract from induced high-renin mice that had been diluted 10-fold (Fig. 2*A*). No precipitin band was observed with the original supernatants from induced or uninduced low-renin animals. When the supernatants from low-renin mice were concentrated 50-fold prior to immunodiffusion, a precipitin band that exhibited a line of identity with that from the high-renin supernatant was observed in preparations from induced but not from uninduced low-renin animals. This result indicates that there is a quantitative difference in immunoreactivity as well as in renin activity between the strains. Furthermore, similar precipitin bands that exhibited a line of identity were observed with the submaxillary gland supernatants from high-renin mice and concentrated supernatants from the kidneys of both high-renin and low-renin mice (data not shown). In contrast, precipitin bands with lines of identity were observed when the induced supernatant from either strain was tested with antibody to nerve growth factor or to epidermal growth factor, two other proteins induced by androgens in the submaxillary gland (6, 7) (Fig. 2*B* and *C*). No precipitin bands were observed when supernatants from uninduced mice of either strain were tested with antibody to nerve growth factor or to epidermal growth factor. These findings indicate that there is no gross difference in the inducibility of these factors in the two strains.

Renin activity was measured in submaxillary glands from induced and uninduced F_1 hybrid females and females from a variety of backcrosses (Fig. 3). In each instance, the level in the F_1 hybrid was between the levels in the parent strains. When the F_1 hybrid was backcrossed with each parental strain approximately half the values resembled those of the parental strain and half were similar to those of the F_1 hybrid. Furthermore, in F_2 hybrids the ratio in the induced animals was roughly 7:14:3 for low, intermediate, and high ranges of activity.

DISCUSSION

Clear-cut differences in basal and androgen-induced renin activity in the submaxillary gland of inbred strains of mice were confirmed. This difference does not appear to be due to differences in endogenous androgen levels between the two strains. In the induced state, no gross differences were demonstrated between the two strains in regard to other androgen-mediated functions such as the response of esteroproteolytic activity, nerve growth factor, or epidermal growth factor. Thus, the variation in renin activity appears to be relatively specific, and the findings in various hybrid animals suggest that this difference between two specific inbred mouse strains (SWR/J and C57BL/10J) is determined by a single gene. Furthermore, the difference in renin activity between the two strains appears to be the result of different amounts of the enzyme rather than of qualitative differences between the two enzymes. Absolute proof of the latter supposition would require amino acid sequencing of the purified proteins from the two strains, but the 100-fold difference in activity of submaxillary gland renin between the induced strains is accompanied by a similar difference in amount of cross-reacting material as determined by immunodiffusion. The fact that no such difference was observed between kidney extracts from the two strains in either renin activity or cross-reacting material supports the concept that the activity differences in submaxillary renin are quantitative; the latter inference is in keeping with the fact that, to date, no differences have been documented between renal and submaxillary gland renins (13, 29). Thus, considered together, the present findings suggest that the difference between SWR/J and C57BL/10J strains is mediated by a regulatory gene rather than by the structural gene for the enzyme.

How such a regulatory gene might act is not clear at present.

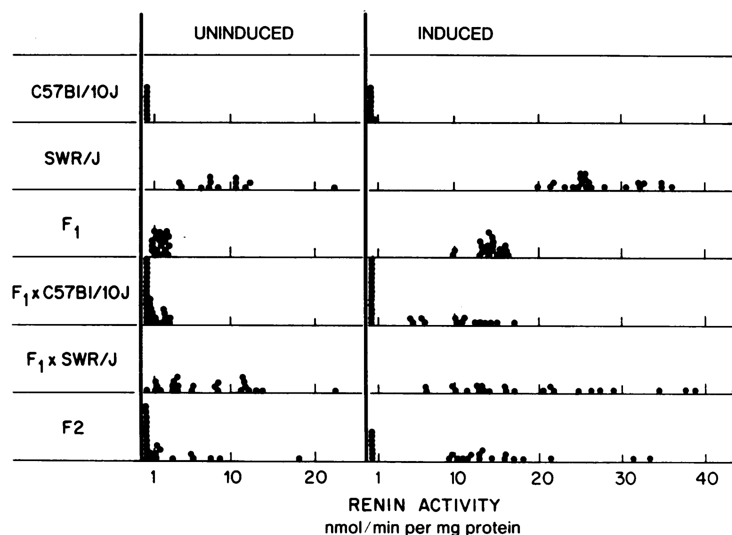

FIG. 3. Genetic analysis of basal and induced renin activities in C57BL/10J, SWR/J, and various crosses. In each instance, 6-week-old female mice were injected either with 0.2 ml of sesame oil on days 0, 2, 4, and 6 and killed on day 7 (uninduced) or with the same amount of sesame oil containing 0.2 mg of dihydrotestosterone (induced). The F_1, backcross, and F_2 data were obtained in females born after reciprocal crosses. Because no differences were observed that depended on the identity of the female parent, the data are pooled.

Because the renin assay is sensitive over a wide range of enzyme activity, it was possible to demonstrate a manyfold induction of activity by androgen in the low-renin strain of mice despite the fact that the induced level of activity was more than 100-fold less in the low-renin than in the high-renin strain. The fact that a high level of activity was present in untreated mice of the high-renin strain and that a significant induction was demonstrated in the low-renin strain makes it impossible to be certain whether the difference between the two strains is in a gene that determines the androgen effect (1) or in some gene that influences the androgen effect secondarily.

It should be pointed out that the regulatory gene for renin cannot be the gene identified by Swank *et al.* (1) that controls the inducibility by androgen of β-glucuronidase in mouse kidney because in that study there was no difference in β-glucuronidase response to androgen in the kidney of SWR/J and C57BL/10J mice.

The tentative identification of another gene that may be concerned with a specific androgen response has important implications in regard to the mechanism of androgen action—namely, that individual, specific regulatory genes may be involved in each cellular action of the hormone. If such is the case, it would provide a means of explaining the striking variability in specific androgen action among individuals and species under circumstances in which overall androgen function is not influenced.

Furthermore, the identification of a regulatory gene for submaxillary renin activity may have equally important implications for understanding the mechanism of control of renin by regulators other than androgens.

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